Ontario Ministry of the Environment

PART D
ANALYTICAL METHODS

NOVEMBER 30 &
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PROCEEDINGS

TECHNOLOGY TRANSFER CONFERENCE

NOVEMBER 30 - DECEMBER 1, 1987

ROYAL YORK HOTEL

PART D

ANALYTICAL METHODS

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INTRODUCTION

Environment Ontario holds its annual Technology Transfer Conference to report and publicize the progress made on Ministry-funded projects. These studies are carried out in Ontario universities and by private research organizations and companies.

The papers presented at the 1987 Technology Transfer Conference are included in five volumes of Conference Proceedings corresponding to the following sessions:

Part A:	Air Quality Research
Part B:	Water Quality Research
Part C:	Liquid & Solid Waste Research
Part D:	Analytical Methods
Part E:	Environmental Economics.

This part is a compilation of papers presented during Session D of the Conference.

For further information on any of the papers, the reader is kindly referred to the authors of to the Research Management Office at (416) 323-4574, 332-4573.

ACKNOWLEDGMENTS

The Conference Committee would like to thank the authors for their valuable contributions to environmental research in Ontario.

DISCLAIMER

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C. Tashiro, R. Clement, M. Lusis, D. Orr and N. Reid, Environment Ontario, Toronto, Ontario.

APPLICATION OF POLYMERIC LIQUID CRYSTAL CAPILLARY COLUMNS FOR SEPARATION OF 2,3,7,8-TCDD AND TCDF BY GC-MS TECHNIQUES

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ABSTRACT:

Since the initial disclosure of the use of polymeric liquid crystal (PLC) stationary phases in capillary column gas chromatography for the separation of polycyclic aromatic hydrocarbons (PAH), extensive work has been carried out on development of such columns for isomer specific separation and quantitation of the recognized carcinogen benzo[a]pyrene in environmental samples. However, PLC capillary columns have not been used for the analysis of PCDD and PCDF.

The linear and planer solute molecules have longer retention as compared to non-linear and bulkier molecules on PLC column. The positive identification and quantitation ofpolychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) in sample matrices that contain high levels of other naturally occurring compounds and other chlorinated industrial pollutants has presented several difficulties. The conventional capillary columns are inadequate for isomer specific (2,3,7,8-TCDD) separation in a congener group and separation of interfering compounds (PCB) from PCDD and PCDF.

This paper will describe the potential of PLC capillary columns for the separation and the identification of PCDD and PCDF. The results of isomer specific separation of 2,3,7,8-TCDD from rest of the tetrachlorodibenzo-p-dioxin isomers will be presented.

INTRODUCTION:

Liquid crystalline compounds as stationary phases have been used in gas chromatography for isomer specific separations (1). The separation mechanism is based on molecular size and shape of the solutes. On liquid crystalline phases linear and planar solute molecules are retained longer than bulkier and non linear solutes of similar chemical and physical properties. The application of high temperature nematic liquid crystals for the separation of three to seven ring polycyclic aromatic hydrocarbon and PCB isomers of environmental importance have been demonstrated (2-6). The isomer-specific separation of positional, geometrical and optical isomers are reported using low molecular weight monomeric liquid crystalline stationary phases (7-10). There are several difficulties in using monomeric liquid crystals in capillary columns to achieve higher efficiency and selectivity (11-13).

Recently, polymeric liquid crystals (PLC) have been developed for use in capillary gas chromatography. Finkelmann first reported the use of liquid crystalline polysiloxane (LCPS) stationary phases in gas chromatography (14). Since then a number of reports have appeared on the applications and development of PLC stationary phases for isomer specific separations (15-21). Lee has developed several LCPS stationary

phases and demonstrated the usefulness of these phases in the analysis of important complex environmental mixtures (22). It has been shown that PLC capillary columns have a high isomer specific selectivity and efficiencies comparable to those of conventional columns.

These highly selective columns should be useful for the separation and quantitation of complex samples containing PCDD, PCDF and PCB. There are 75 PCDD and 135 PCDF isomers. Separation of those isomers from other compounds present in complex environmental samples is very difficult. In particular, separation of these compounds from high concentrations of PCB is challenging. Selectivity based on molecular weight can be achieved using the GC-MS/EISIM mode for the detection of PCDD and PCDF from co-eluting compounds of different molecular weight. However, the fragment ions of certain co-eluting compounds such as PCB interfere in the analysis of PCDD and PCDF. If interfering compounds can be separated prior to their analysis by GC-MS/EISIM technique positive identification and quantitation of PCDD and PCDF is possible using various criteria (23).

This paper shows the unique selectivity of LCPS capillary column for the separation of 2,3,7,8-TCDD and TCDF from other tetra isomers. The separation of large number of tetra- to octa-chloro dioxins and dibenzofuran isomers is compared with the DB-5

column. The quantitative analysis of 2,3,7,8-TCDD and TCDF and all PCDD and PCDF in an extract of flyash from a municipal incinerator in a single GC-MS run and separation of 2,3,7,8-TCDD and TCDF from sample with high PCB content accomplished on an LCPS column.

EXPERIMENTAL SECTION:

The Gas Chromatograph used was a Hewlett Packard model 5880, equipped with electron capture dector(ECD) and a cool-on column injector. The GC-MS system used was a Hewlett Packard 5987 with an HP1000 data system, cool-on column injector, and splitless direct interface between the GC and MS.

Flyash samples were soxhlet extracted for 48 hours using benzene. Benzene extracts were concentrated to 5 to 25 ul/gram flyash. In the GC-MS/EISIM analysis, the ion source temperature was 200 C. The ions monitored for the tetra to octa chlorodioxins and furans were M, M+2, M+4 or M+2, M+4, M+6 for each congener group. The retention windows were determined by analysis of a Ontario flyash extract that contained all PCDD and PCDF. Selectivity of column for separation of 2,3,7,8-TCDD and TCDF was confirmed from retention time of standards. The quantitation of 2,3,7,8-tetra chlorodibenzodioxin and furan isomers was carried out using external standards. For the

quantitation of total PCDD and PCDF congeners an external standard containing one isomer of each congener group from tetra- to octa-chloro dioxins and dibenzofurans was used. Concentrations of all PCDD and PCDF standards were from 100 to 500 pg/ul.

RESULTS AND DISCUSSION:

Analytical methodology can be simplified if a gas chromatographic column can separate the interfering compounds, as well as, the isomers in all congener groups. Figure 1 and 2 contrast the separation of the tetra- to octa- chlorodibenzo-dioxins in a flyash extract on a liquid crystalline polysiloxane (LCPS) column with the DB-5 column. A similar contrast for the separation of the tetra- to octa- chlorodibenzofurans in the flyash extract is shown in figure 3 and 4. Identification of PCDD and PCDF congeners was carried out by monitoring three characteristic ions such as M, M+2, M+4 for the tetra to hexa chloro substituted congeners and M+2, M+4, M+6 for the hepta and octa chloro substituted congeners. The peaks present in the three characteristic ions, for a congener group, in proper intensity ratios and retention windows were the identification criteria used for the respective congener group isomers. Mass

chromatograms of the most intense ions of each congener group are shown in figures 1 to 4. The separation on the LCPS column is clearly different than that on the DB-5 column. Retention behaviour on the non polar DB-5 column is based on the boiling points of the solute molecules. The separation of large number of PCDD and PCDF isomers in a congener group could not be observed on the DB-5 column presumably due to their similar boiling points. Separation on the LCPS stationary phase depends on the shape, polarity and the boiling points of the solute molecules. This gives a pronounced separation of several isomers in a congener group on the LCPS column. The separations of large number of isomers in congener groups observed on the LCPS column are comparable to that on long polar columns (24, 25).

The toxicity of individual isomers of PCDD and PCDF depends upon the degree of chlorination and position of chlorine substitution on the dibenzodioxin and dibenzofuran structures. Because of difficulties in separation and non availability of several isomers, the quantitative results are presented as the total amount of all the isomers present in a congeners group. However, the true assessment of toxicity of a particular sample depends upon the separation, positive identification and quantitation of the most toxic isomers. Tetrachlorodibenzodioxins (TCDD) and tetrachlorodibenzofurans (TCDF) have shown higher

toxicity than the rest of the isomers, in particular 2,3,7,8-TCDD and 2,3,7,8-TCDF are recognized as the most toxic isomers from the animal tests. The study of the retention behaviour and separation of 2,3,7,8- TCDD and TCDF using very long capillary columns with different stationary phases shows that a separation of 7 to 17 out of 22 TCDD isomers and 28 out of 38 TCDF isomers can be achieved on different columns (23). The LCPS capillary column used in this study shows separation of 16 TCDD and 27 TCDF isomers present in the flyash extract. It is clear from the figures 1 and 3 that the LCPS column can give isomer specific separation of 2,3,7,8-TCDD and TCDF, as well as, the penta- to octa-chloroisomers. Selectivity shown by the LCPS column for 2,3,7,8-TCDD and TCDF is unique. In all the tetra isomers,the 2,3,7,8-TCDD isomer has a highly symmetrical structure and a large length to breadth ratio. Thus, based on the mechanism of separation on liquid crystal stationary phases, it is retained longer than other tetra isomers. Similarly, the longer retention time and separation of 2,3,7,8-TCDF from all tetrachlorofuran isomers can be explained. The separations of the most toxic isomers were confirmed by spiking the flyash extract with carbon-13 labelled 2,3,7,8-TCDD and TCDF. Their mass chromatograms are shown in figure 1 and 3.

A comparative study of quantitative analysis of PCDD and

PCDF in flyash samples from the Paris Tiru incinerator (France), Hiroshima incinerator (Japan) shows that the results obtained on the LCPS column and DB-5 columns are comparable. However the LCPS column, in addition gave quantitative analysis of the most toxic 2,3,7,8-TCDD and TCDF isomers in the same run. It is interesting to note that 2,3,7,8-TCDF elute after 2,3,7,8-TCDD on polar columns(24). However, on the LCPS , DB-5 and OV-101 columns retention order is reverse. The LCPS column provide a simple and fast analytical technique for separation of PCDD, PCDF and the most toxic 2,3,7,8-TCDD and TCDF isomers in complicated environmental samples.

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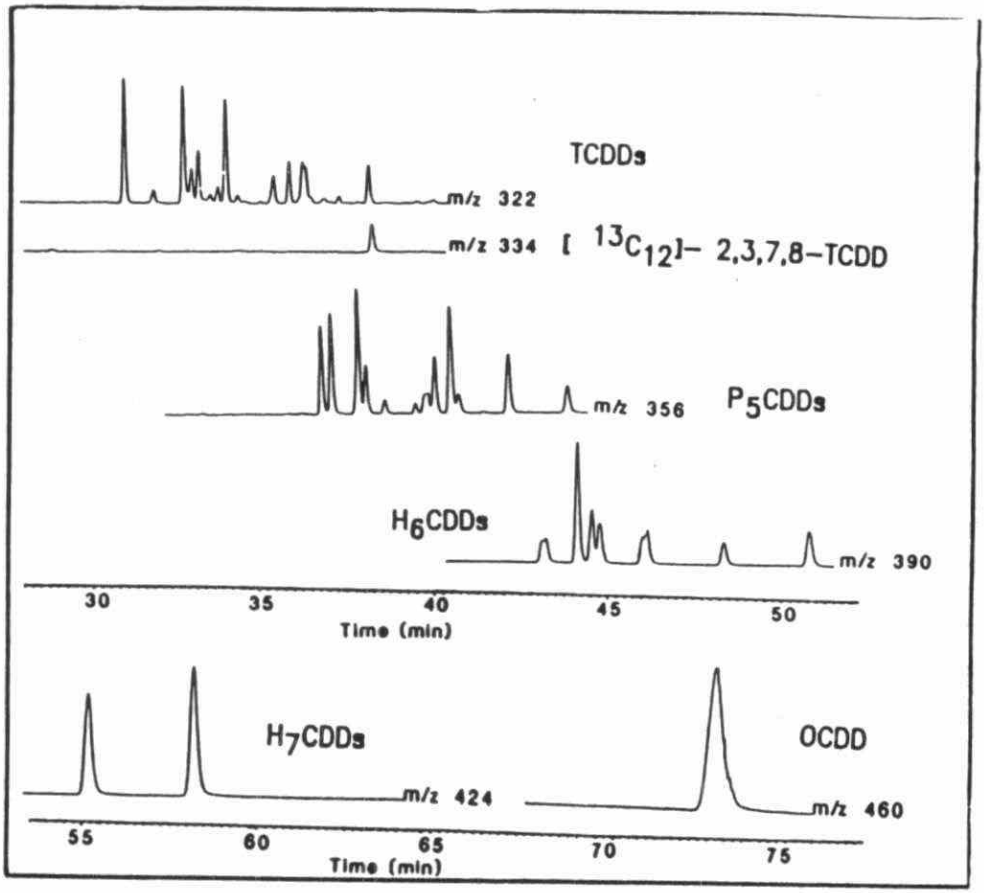

Figure 1. Separation of PCDDs in Ontario flyash extract: mass chromatograms of m/z 322 (TCDDs), m/z 334 [$^{13}C_{12}$-2,3,7,8-TCDD], m/z 356 (P_5CDDs), m/z 390 (H_6CDDs), m/z 424 (H_7CDDs), m/z 460 (OCDD). Chromatographic conditions were as follows: 20 m X 0.25 mm i. d. LCPS fused silica column; temperature at 80 ^{0}C for 1 min, programmed to 245 ^{0}C at 4 ^{0}C/min, then to 280 ^{0}C at 2 ^{0}C/min, 30 min at 280 ^{0}C.

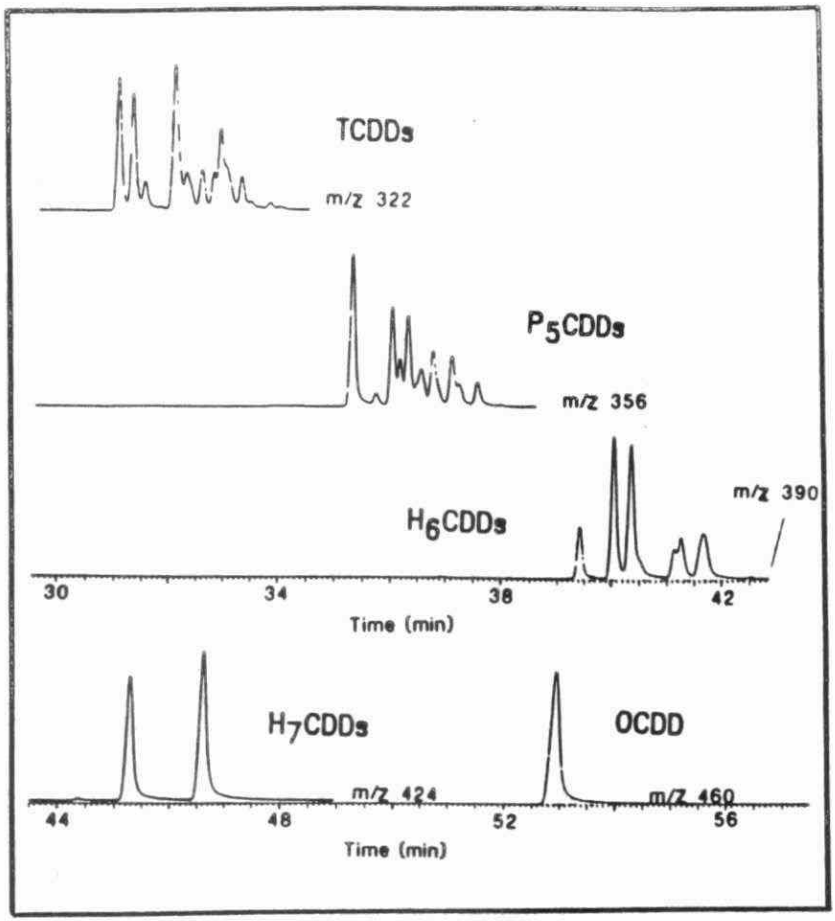

Figure 2. Separation of PCDDs in Ontario flyash extract: mass chromatograms of m/z 322 (TCDDs), m/z 356 (P_5CDDs), m/z 390 (H_6CDDs), m/z 424 (H_7CDDs), m/z 460 (OCDD).
Chromatographic conditions were as follows:
30 m X 0.32 mm i. d. DB-5 fused silica column; temperature at 80 ^{0}C for 1 min, programmed to 300 ^{0} C at 4 ^{0}C/min, 5 min at 300 ^{0}C.

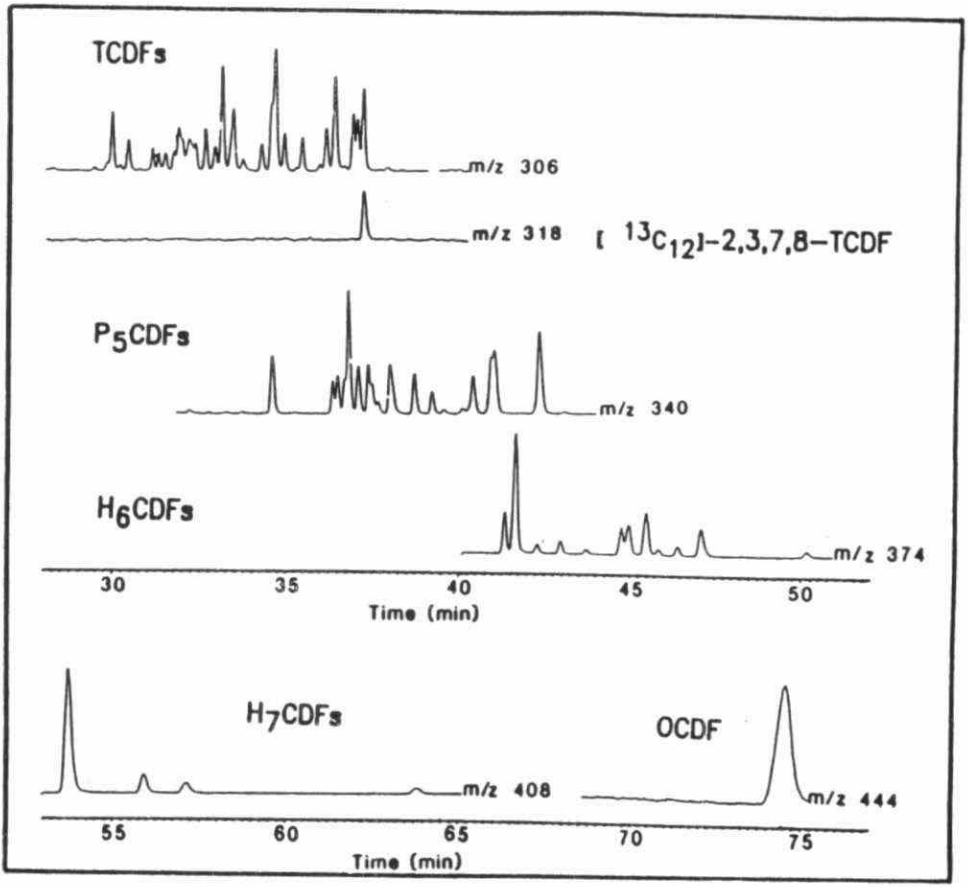

Figure 3. Separations of PCDF in Ontario flyash extract: mass chromatograms of m/z 306 (TCDFs), m/z 318 [$^{13}C_{12}$-2,3,7,8-TCDF] m/z 340 (P_5CDFs), m/z 374 (H_6CDF), m/z 408 (H_7CDF), m/z 444 (OCDF).

Chromatographic conditions were as follows:

20 m X 0.25 mm i. d. LCPS fused silica column; temperature at 80 ^{0}C for 1 min, programmed to 245 ^{0}C at 4 ^{0}C/min, then to 280 ^{0}C at 2 ^{0}C/min, 30 min at 280 ^{0}C.

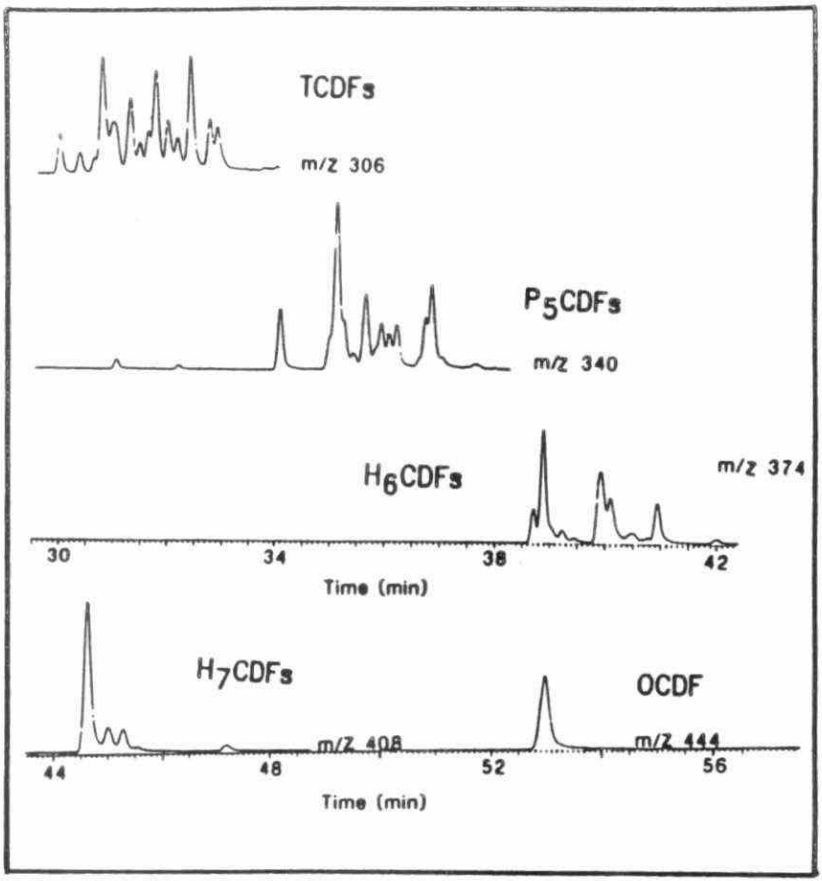

Figure 4. Separations of PCDF in Ontario flyash extract: mass chromatograms of m/z 306 (TCDFs), m/z 340 (P_5CDFs), m/z 374 (H_6CDF), m/z 408 (H_7CDF), m/z 444 (OCDF).
Chromatographic conditions were as follows:
30 m X 0.32 mm i. d. DB-5 fused silica column; temperature at 80 ^{0}C for 1 min, programmed to 300 ^{0} C at 4 ^{0}C/min, 5 min at 300 ^{0}C.

Evaluation of Monitoring Techniques for Dioxins in Ambient Air

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ABSTRACT

Chlorinated dibenzo-p-dioxins (CDDs) and dibenzofurans (CDFs) in ambient air have not been widely investigated. A few measurements of 2,3,7,8-tetra chlorodibenzo-p-dioxin (2,3,7,8-4CDD) in ambient air using high volume air sampling with glass fibre filters and polyurethane foam (PUF) cartridges have been reported. However, no validated methodologies for sampling ambient air for the full range of CDDs/CDFs have been presented. Proper validation requires spiking studies of multiple CDD/CDF congener to determine breakthrough of both the glass fibre filter and the PUF cartridges, and recovery studies to determine extraction efficiencies.

The Ontario Ministry of the Environment ambient air sampler for CDDs/CDFs consists of a glass fibre filter followed by a single or dual PUF cartridges. Low and high-level CDD/CDF surrogate spiking studies have been performed to determine the sampling efficiency of this device. ^{13}C-labelled standards were spiked separately onto the glass fibre filter and PUF cartridges to determine breakthrough.

Initial results from a 24 hour sampling with a glass fibre filter and a single PUF indicated good spike recoveries for both the PUF and filter and some breakthrough of the lower congener spikes from the filter to the PUF.

INTRODUCTION

Interest in the analysis of CDDs and CDFs has increased over the past few years due to the knowledge of the toxic nature of these compounds. The methodology for the sampling and analysis of CDDs and CDFs in samples such as drinking water, fish, soils, sediments, effluents and stack emissions has been well developed. However, the analysis of dioxins and furans in ambient air has not been widely investigated because of the need for very low detection limits (pg/m^3). Another factor in the slow development of ambient air methodology is the selection of suitable sampling apparatus. CDDs and CDFs can exist in ambient air in two forms: 1) adsorbed on particulate matter and 2) as vapour phase molecules. It is also known that the concentrations found in either form may not necessarily be related to one another. One must be able to sample either the particulate, the vapour phase or both, as desired.

A number of sampling devices have been developed for monitoring ambient CDDs and CDFs. Most encorporate a particulate filter and an adsorbent material to trap vapour phase molecules, in series, attached to a vacuum source. Smith et.al.used a glass fibre filter, followed by silica gel in a removable cartridge (1). Silica gel was chosen as an adsorbent because it is easily cleaned and easily handled. Smith was able to obtain detection limits of 0.003 pg/m^3 for 2,3,7,8-TCDD but did not look at the full range of dioxin and furan congeners.

One of the more widely used adsorbents in ambient air monitoring is the polyurethane foam (PUF) plug. PUF is the material used in the upholestry industry and is inexpensive and convenient to handle. The PUF plug has been used by the U.S. Environmental Protection Agency to monitor 2,3,7,8-TCDD levels in ambient air(2). A glass fibre filter and a PUF plug housed in a General Metal Works, Inc., Model PS-1 air sampler was used. In laboratory spiked samples, 95% recovery of 2,3,7,8-TCDD was obtained, indicating the validity of the method for field samples(2). PUF adsorbents have also been used to monitor other types of volatile organic compounds such as pesticides (3,4), polycyclic aromatic hydrocarbons (4) and polychlorinated biphenyls (4) at the ng/m^3 level.

The Ontario Ministry of the Environment, Laboratory Services Branch and Air Resources Branch, has been evaluating one monitoring technique for the determination of total dioxin and total furan congeners in ambient air. The Ministry used a modified Hi-volume sampler with a glass fibre filter and single or dual PUF cartridges. Spiking experiments were carried out using ^{13}C-labelled dioxin and furan standards. The filters and PUF cartridges were spiked with different labelled congeners. The samplers were set up and air was drawn through the filter and PUF for extended periods of time. At the end of the sampling period, the filters and PUF cartridges were spiked with an additional labelled congener, extracted, cleaned and analyzed by GC/MS. Locations of the labelled congeners and their levels indicated if breakthrough from the filter to the PUF cartridge or from the PUF cartridge occurred and also indicated extraction efficiencies. High and low level spiking experiments were carried out with both single and dual PUF cartridges.

The results of these experiments are aiding the Ministry to develop both a sampling and analytical protocol that will later be evaluated under field conditions. Further feasibility and precision experiments will be required before a protocol can be determined.

EXPERIMENTAL

Materials - Glass fibre filters were obtained from Pallflex Products Corp. (Putnam, Conn.). Polyurethane foam was purchased from a local upholestry manufacturer. Distilled-in-glass grade solvents were used (Caledon Laboratories, Georgetown, Ontario). ^{13}C-labelled dioxin and furan standards were obtained from Cambridge Laboratories (Cambridge, MA.).

Apparatus - A modified Hi-Volume sampler with a single glass fibre filter and single or dual PUF cartridges was used. The samplers were assembled and spiked in the laboratory. A range of ^{13}C-labelled dioxin and furan standards were spiked on the filters and cartridges. Both high level (1-long/ congeners) and low level (300-500 pg/congener) spikes were performed. Sampling was carried out for 24 hours in the case of a single PUF cartridge and for 72 hours when 2 PUF cartridges in series were used. Temperature and flow-rate data were monitored during the test period. Field blanks samples were also taken.

The aluminum filter housing and cartridge housings were cleaned with distilled water and solvents prior to usage. The PUF cartridges were cleaned before sampling by Soxhlet extraction with toluene for 24 hours. The cartridges were rinsed with methylene chloride and proven clean. The PUF cartridges were stored in clean aluminum foil prior to use.

Analysis - After sampling, the filters and cartridges were stored in pre-cleaned aluminum foil in a refridgerator prior to extraction. The filters and cartridges were extracted separately by Soxhlet extraction, with toluene for 24 hours. Prior to extraction the samples were spiked with ^{13}C-6CDD as an internal standard to provide extraction efficiencies. The extracts were concentrated to approximately 5-10 mL using a rotary evaporator. The dioxin/furan containing fractions were subjected to a modified Dow cleanup using NaOH/Silica, H_2SO_4/Silica, $AgNO_3$/Silica and activated alumina adsorbents. The final fraction was taken to dryness under a gentle stream of nitrogen and submitted for GC/MS analysis.

The GC/MS analysis was carried out using a Finnigan 4000 GC/MS/DS (Sunnyvale, CA.) in the selected ion monitoring mode. All ^{13}C-labelled congeners used were monitored in both the filter and cartridge samples. Percentage recoveries and breakthroughs of the congeners from the filters and from the cartridges were calculated.

Results and Discussion

An initial high level spike experiment using a glass fibre filter and a single PUF cartridge was carried out December 23, 1986. The spike recoveries for the filters are shown in Table 1 and for the cartridges are shown in Table 2. The samples were spiked with labelled dioxins (^{13}C-5CDD and ^{13}C-7CDD on the filters and ^{13}C-4CDD and ^{13}C-8CDD on the PUF cartridges) and were collected for a 24 hour period. The average sampling volume was 1800 m^3. The recovery of ^{13}C-5CDD on the PUF indicated that there was some breakthrough of the lower congener from the filter onto the PUF cartridge.

Good recoveries for the other spiked congeners on both the filters and cartridges were observed for the field samples. The low spike recovery for the field blank samples can be partially accounted for by the low recovery of the ^{13}C-6CDD internal standard. Good detection limits (0.02 pg/m^3 for 4CDD and 4CDF to 1 pg/m^3 for 8CDD and 8CDF) were obtained for the field samples. These detection limits are lower than those required if one accepts the New York State Health Department recommendation of an acceptable daily intake of 2 (pg/Kg)/day for 2378-TCDD in humans(5).

A further set of four experimental ambient air collections was carried out as follows:

Sample 1: Filter + Single PUF + High Level Spike (24 hours)

Sample 2: Filter + Single PUF + Low Level Spike (24 hours)

Sample 3: Filter + Dual PUFs + High Level Spike (72 hours)

Sample 4: Filter + Dual PUFs + :pw :eve; S^{3}ple)72 hours)

These samples were collected over the period from May 1987 to July 1987. ^{13}C-4CDD, ^{13}C-8CDD, ^{13}C-5CDF and ^{13}C-7CDF were spiked on the filter and ^{13}C-5CDD, ^{13}C-7CDD, ^{13}C-4CDF and ^{13}C-8CDF were spiked on the PUF cartridges of samples 2, 3, and 4. Sample 1 spikes were reversed. ^{13}C-6CDD was used as an extraction efficiency spike. Detection limits of 0.02 pg/m^3 0.05 pg/m^3 for the lower congeners (4CDD & 4CDF) and 0.08 pg/m^3 to 0.7 pg/m^3 for the higher congeners (8CDD & 8CDF) were obtained.

Low recoveries of the tetra- and penta- congeners and higher recoveries of the hexa- to octa- congeners were observed for the filter samples from all four field runs. In sample 1 (Single PUF/High Spike/24 hours collection) 50-100% recovery of the congeners spiked on the PUF was obtained. There was higher recovery of the hepta- and octa- congeners than of the more volatile tetra- and penta- congeners. The PUFs were also monitored for the congeners that were originally spiked on the filters. High levels for the 4CDF and 5CDD were observed, indicating a high degree of breakthrough from the filter to the PUF. There was a lesser degree of breakthrough from the PUF cartridge.

Lower recovery of PUF spikes were obtained for sample 2 (Single PUF/Low Spike/24hr collection). ^{13}C-8CDF was not observed on the PUF at the low spike levels used. Breakthrough of the labelled congeners from the filters onto the PUFs was also observed.

One conclusion that can be drawn from the results for the single PUF experiments is that the MOE sampler allows the determination of ambient dioxins and furans in both the particulate state and vapour phase. It can also be concluded that a single PUF cartridge may be adequate for ambient air monitoring as there is no significant breakthrough from the cartridge. The lower recoveries obtained in sample 2 for the labelled congeners were not a breakthrough problem but a detection limit problem. The spike levels used were close to the detection limits and losses in extraction and cleanup could have dropped the levels below the detection limit.

Future work involves carrying out feasibility and precision studies of the sampling and analysis methodology in various locations. Preliminary results indicate that a glass fibre filter and single PUF cartridge will be used. The Ministry is also involved in an interlab comparison with Environment Canada that will enable development of a protocol for monitoring CDDs and CDFs in ambient air in Ontario.

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TABLE 1: % Spike Recoveries-Filter Samples (High Spike-1 PUF)

Isomer	Field Sample A	Field Sample B	Field Blank
^{13}C-5CDD(A)	78	66	26
^{13}C-7CDD(A)	140	110	44
^{13}C-6CDD(B)	90	64	24
^{13}C-4CDD(C)	NS	NS	NS
^{13}C-8CDD(C)	NS	NS	NS

A = spiked on filter
B = spiked just prior to extraction
C = spiked on PUF
NS= not spiked

TABLE 2: % Spike Recoveries - PUF Samples (High Spike - 1 PUF)

Isomer	Field Sample A	Field Sample B	Field Blank
^{13}C-4CDD(C)	88	63	3
^{13}C-8CDD(C)	130	110	52
^{13}C-6CDD(B)	120	96	48
^{13}C-5CDD(A)	15*	13*	9*
^{13}C-7CDD(A)	NS	NS	NS

A = spiked on filter
B = spiked just prior to extraction
C = spiked on PUF
NS= not spiked
* = not spiked but detected on PUF

ANALYSIS OF DIOXINS, FURANS AND OTHER POLYCHLORINATED POLLUTANTS IN FISH

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Many of the open column chromatographic methods used for the cleanup of fish tissue extracts employ alumina packing for the separation of PCDDs and PCDFs from PCBs and other chlorinated pollutants. Based on this fact, an HPLC column containing alumina packing material was prepared. A new cleanup procedure was devised in which the HPLC fractionation on the alumina column replaces the multi-step open column chromatographic method employed in the Dow cleanup. The new method allows for the simultaneous determination of PCDDs, PCDFs, and other polychlorinated pollutants of interest.

ANALYSIS OF DIOXINS, FURANS, AND OTHER POLYCHLORINATED POLLUTANTS IN FISH

INTRODUCTION:

The gas chromatograph/mass spectrometer is a powerful tool for performing the analysis of complex organic samples. It sometimes however lacks the selectivity necessary to unambiguously distinguish between certain species present in the matrix. As a result quantitation of desired components may be impossible or unreliable. This is especially true when the compounds of interest are present at low levels relative to the interfering components.

In recent years polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) have become a very controversial concern to both scientists and citizens. It is critical that the levels reported are not the result of false positives or negatives. The former occurs when insufficient separation of the PCDDs and PCDFs from interfering matrix components leads to higher reported levels than actually present. False negative results will occur if some of the dioxins or furans are lost in the sample preparation procedure. A sample cleanup method must clearly be both efficient and reproducible in order that the reported data be significant.

The fractionation of complex environmental matrices such as fish tissue extracts often involves the use of open column chromatography. A variety of different column packing materials have been employed for numerous types of

separations. The separation of polychlorinated biphenyls (PCBs) from PCDDs and PCDFs using column chromatographic procedures has been reported in the literature (1-7). In many of the studies carried out, column chromatography with alumina packing materials was found to provide a reasonable separation of dioxins and furans from components in the sample matrix, including PCBs, which interfere in their determination.

High performance liquid chromatography (HPLC) is being increasingly used as a cleanup procedure prior to gas chromatographic analysis. HPLC has numerous advantages over conventional liquid chromatography (LC). Perhaps most importantly, HPLC columns are much more efficient than open columns used in LC. Higher reproducibility in elution behaviour is observed in HPLC because the same column is used repeatedly. The HPLC column characteristics remain relatively constant provided reasonable care is taken such that no particulate matter is introduced into the system and no sample components are left retained on the stationary phase. The time necessary to achieve the desired separation is usually much faster by HPLC. The application of automated sample injection and fraction collection increases the sample throughput and permits the chemist to devote his attention to other tasks. A wide variety of on-line detection systems and the implementation of microprocessor controlled mobile phase gradient elution programming have also resulted in the routine application of HPLC as a cleanup procedure.

Despite all of the advantages of HPLC over LC,

reported separations of PCDDs and PCDFs from PCBs have been almost exclusively accomplished by open column procedures. In fact, the use of alumina packing material which has been so successfully applied in LC separations has seen limited use in HPLC columns. Dolphin and Willmott report the separation of dioxins from other chlorinated congeners (8). In this work they assume that the HPLC retention window for all of the PCDD isomers can be bracketed by the nonchlorinated dibenzo-p-dioxin and the fully chlorinated PCDD, octachlorodibenzo-p-dioxin (OCDD). The separation of these two dioxins from PCBs and polychloronaphthalenes (PCNs), specifically Aroclor 1268 and Halowax 1051 respectively, plus the pesticide p,p'-DDE is reported. No mention is made regarding the relative concentrations of the components injected upon the HPLC nor the efficiency of the method for separating a relatively large amount of either PCBs or PCNs from the dioxins. The use of dibenzo-p-dioxin and OCDD as retention window markers was not validated by injecting a mixture known to contain most or all of the 75 possible PCDD isomers. Although this work seemed to indicate the potential usefulness of alumina packing material in HPLC for the separation of PCDDs from PCBs, more detailed studies were not reported.

In the work carried out under this project, we have further investigated the applicability of HPLC using an alumina column for the separation of complex mixtures of chlorinated pollutants.

EXPERIMENTAL:

An HPLC system consisting of three Waters Model 510 dual piston pumps and a Waters 820 Chromatography Work Station was used to performed all separations. A variable wavelength (Waters Model 481) ultraviolet detector and a six-port Rheodyne injector with a 20 microlitre loop were employed. HPLC fractions were automatically collected using a Gilson Model 202 fraction collector. An alumina HPLC column was prepared in our laboratory using a Shandon column packing apparatus. The column (25 x 0.4 cm i.d.) was packed with a slurry containing 5 micron alumina particles under a pressure of approximately 9000 psi. All solvents used in this study were pesticide grade (distilled in glass) and were filtered prior to use as HPLC mobile phases. The HPLC gradient elution program and fraction collection times are listed in figure 1 and table I respectively.

Gas chromatographic analyses were performed on a Hewlett-Packard HP5880A GC equipped with a ^{63}Ni source electron capture detector (ECD) and flame ionization detector (FID). A cool on-column injector and a 30 metre DB-5 fused silica capillary column (0.32 mm i.d. and 0.25 micron stationary film thickness) were used for all analyses. The GC parameters are summarized in table II.

GC/MS analyses were carried out using a Hewlett-Packard HP5987A GC/MS system having electron impact and chemical ionization capabilities. Mass spectral analyses were performed using two different ionization modes, electron

TABLE I

NORMAL PHASE HPLC PARAMETERS

Injection Loop Volume:	20 microlitres
Column:	5 micron alumina (25 x 0.4 cm)
Mobile Phases:	hexane and dichloromethane
Detector:	ultraviolet (254 nm)

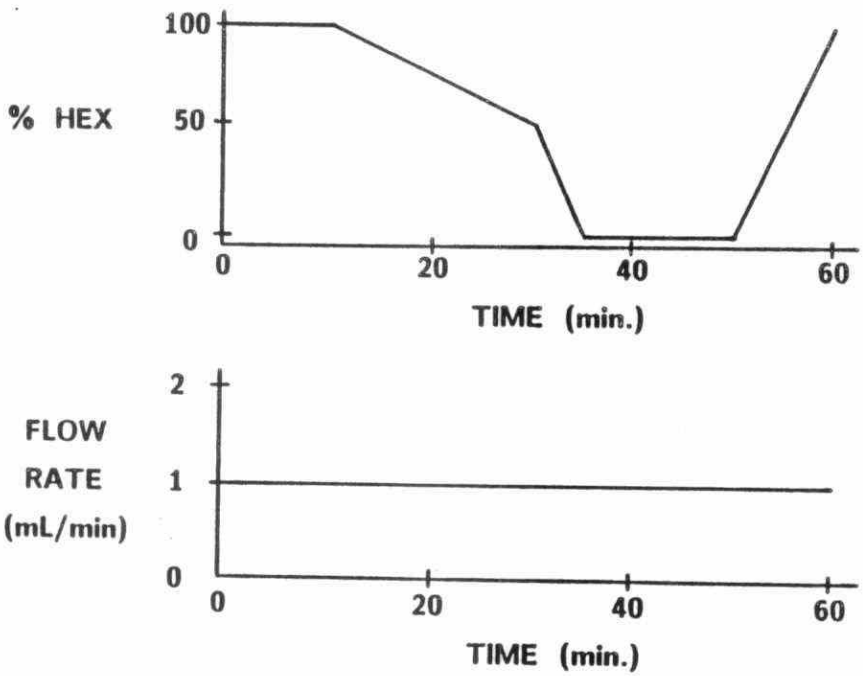

FIGURE 1. HPLC Gradient Elution Program

TABLE II

GAS CHROMATOGRAPHIC PARAMETERS

Detector	FID	ECD
Detector Temperature	350 °C	325 °C
Attenuation	2^3 (8)	2^{11} (2048)
Carrier Gas	helium	helium
Detector Makeup Gas	-	95:5 argon: methane
Initial Oven Temperature	60°C	60 °C
Oven Temperature Program Rate	5°C/min	5 °C/min
Final Oven Temperature	300 °C	300 °C
Final Hold Time	20 min	20 min

impact (EI) and negative chemical ionization (NCI), for both full scan and selected ion monitoring (SIM) data acquisition techniques. Full scan mass spectra were obtained for both ionization modes in order to identify the components collected in the individual HPLC fractions. SIM analyses were necessary to permit the determination of any PCDDs or PCDFs which may be present at ultratrace levels. The increased responses of PCDDs and PCDFs to electron capture NCIMS (relative to EIMS) was exploited in the NCI SIM analyses. The chromatographic column and injector used for all GC/MS runs are identical to those previously described for the GC-ECD and GC-FID analyses. The GC/MS parameters are summarized in table III.

The sample preparation procedure currently used by the Ontario Ministry of the Environment for the determination of dioxins and furans in fish tissue is outlined in figure 2. In this study we have replaced the multi-step open column chromatographic cleanup procedure with a single LC cleanup followed by an HPLC fractionation step prior to GC/MS analysis. The cleanup scheme employed in this study is illustrated in figure 3.

In order to determine the elution window of PCDDs and PCDFs on normal phase HPLC using an alumina column, a mixture of dioxin and furan isomers must be injected on the system. Flyash is believed to contain all PCDD and PCDF isomers and therefore a flyash extract was used to verify the elution behaviour of the dioxins and furans on the alumina column

TABLE III

GC/MS PARAMETERS

A. FULL SCAN ANALYSES:

Mass Spectrometer Parameters

Ionization mode	EI	NCI
Ion Source Pressure	5×10^{-6} torr	1.6 torr
Reagent Gas	-	methane
Emission Current	400 uA	400 uA
Electron Energy	70 eV	255 eV
Repeller Potential	12.60 V	12.75 V
Drawout Potential	34.0 V	39.0 V
Ion Focus Potential	140 V	255 V
Entrance Lens Potential	119 V	140V
X-Ray Lens Potential	70 V	179 V
Electron Multiplier Potential	2196 V	2196 V
Scan Range	50-600 amu	30-600 amu

GC Parameters

Initial Oven Temperature	60 ^{o}C	60 ^{o}C
Oven Temperature Program Rate	5 ^{o}C/min	5 ^{o}C/min
Final Oven Temperature	300 ^{o}C	300 ^{o}C
Final Hold Time	20 min	20 min
Carrier Gas	helium	helium

TABLE III
(cont'd)

GC/MS PARAMETERS

A. SELECTED ION MONITORING ANALYSES:

Mass Spectrometer Parameters

Ionization mode	EI	NCI
Ion Source Pressure	5×10^{-6} torr	1.6 torr
Reagent Gas	-	methane
Emission Current	400 uA	400 uA
Electron Energy	70 eV	255 eV
Repeller Potential	12.75 V	12.75 V
Drawout Potential	37.5 V	127.5 V
Ion Focus Potential	125 V	255 V
Entrance Lens Potential	135 V	175 V
X-Ray Lens Potential	46 V	44 V
Electron Multiplier Potential	2196 V	2196 V
Number of SIM Groups	5	5
Dwell Time	50 msec	50 msec
Group #1 Scan Start	26.0 min	26.0 min
Group #1 Scan Stop	31.1 min	31.1 min
TCDF Ions Monitored	241, 304, 306, 308 amu	304, 306, 308 amu
TCDD Ions Monitored	257, 320, 322, 324 amu	320, 322, 324 amu
Group #2 Scan Start	31.1 min	31.1 min
Group #2 Scan Stop	36.5 min	36.5 min
P_5CDF Ions Monitored	277, 338, 340, 342 amu	338, 340, 342 amu

TABLE III
(cont'd)

P_5CDD Ions Monitored	293, 354, 356, 358 amu	354, 356, 358 amu
Group #3 Scan Start	36.5 min	36.5 min
Group #3 Scan Stop	40.0 min	40.0 min
H_6CDF Ions Monitored	311, 372, 374, 376 amu	372, 374, 376 amu
H_6CDD Ions Monitored	327, 388, 390, 392 amu	388, 390, 392 amu
Group #4 Scan Start	40.0 min	40.0 min
Group #4 Scan Stop	43.0 min	43.0 min
H_7CDF Ions Monitored	345, 408, 410, 412 amu	408, 410, 412 amu
H_7CDD Ions Monitored	361, 424, 426, 428 amu	424, 426, 428 amu
Group #5 Scan Start	43.0 min	43.0 min
Group #5 Scan Stop	58.0 min	58.0 min
OCDF Ions Monitored	381, 442, 444, 446 amu	442, 444, 446 amu
OCDD Ions Monitored	397, 458, 460,462 amu	458, 460, 462 amu

<u>GC Parameters</u>

Initial Oven Temperature	60 ^{o}C	60 ^{o}C
Oven Temperature Program Rate	5 ^{o}C/min	5 ^{o}C/min
Final Oven Temperature	300 ^{o}C	300 ^{o}C
Final Hold Time	20 min	20 min
Carrier Gas	helium	helium

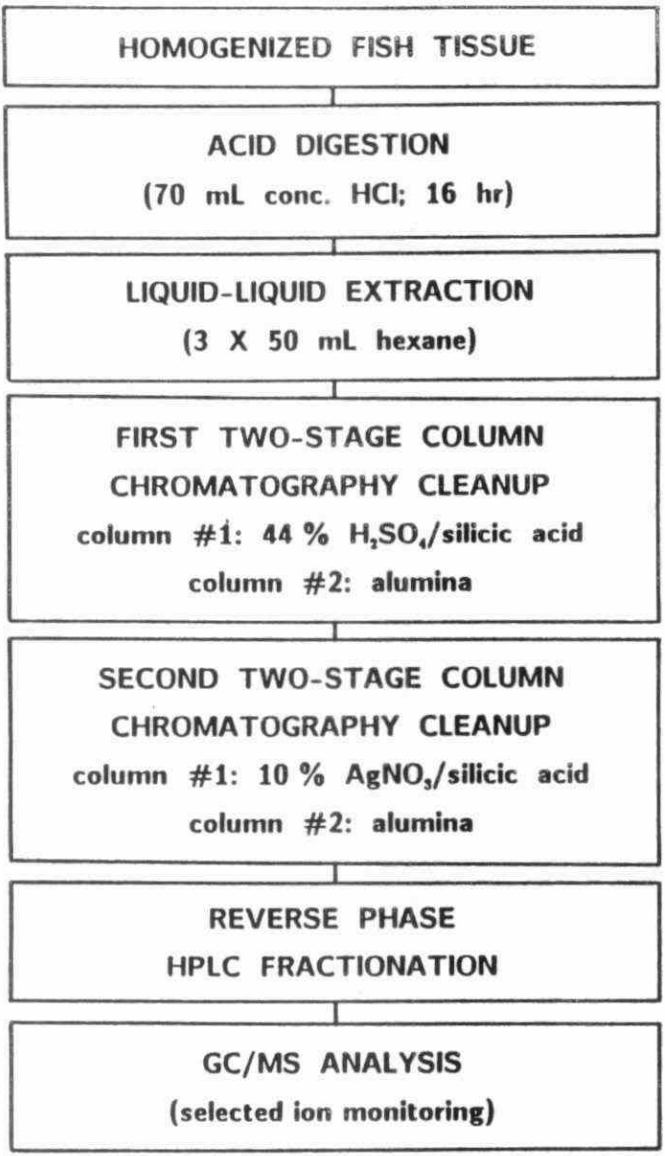

FIGURE 2: Sample cleanup procedure currently used by the Ministry of the Environment

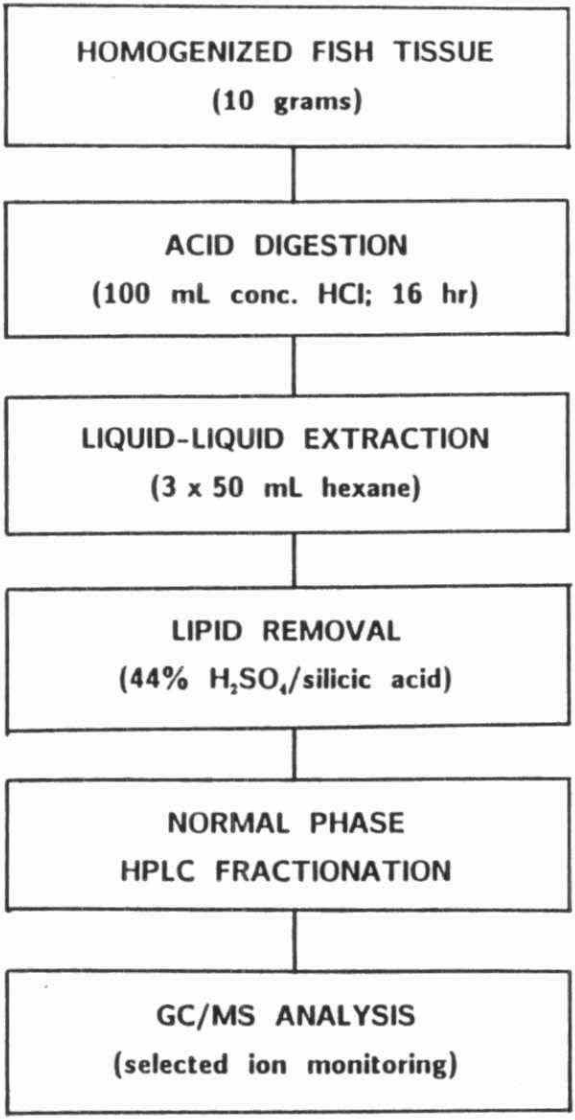

FIGURE 3: Sample cleanup procedure employed in this study

RESULTS AND DISCUSSION:

A flyash extract was injected on the HPLC and a single fraction was collected from 16 to 22 minutes. The SIM profiles of the crude flyash extract and the fraction collected from the HPLC are shown in figure 4. Comparison of the individual ion profiles for the tetra- through octachloro congener groups of PCDDs and PCDFs showed that all the dioxins and furans were collected within this fraction.

A fish tissue sample was carried through the cleanup procedure illustrated in figure 3 and three HPLC fractions were collected over the intervals described in table I. Figures 5 to 10 show the GC traces obtained for the three fractions using flame ionization and electron capture detection.

Each fraction was analyzed by GC/EIMS and GC/NCIMS in the full linear scan mode. The compounds identified in fractions 1 and 2 using these techniques are given in tables IV and V. No compounds could be identified in fraction 3, however based on their NCI mass spectra which showed the presence of Cl^- due to dissociative electron capture, ten of the components were found to be chlorinated.

Fraction #2, corresponding to a collection interval from 16 to 22 minutes, was subjected to SIM analysis in order to determine whether dioxins or furans could be detected. The total ion profiles obtained from the GC/EIMS and GC/NCIMS SIM analyses are illustrated in figure 11. A component eluting from the GC is identified as a dioxin or furan only if

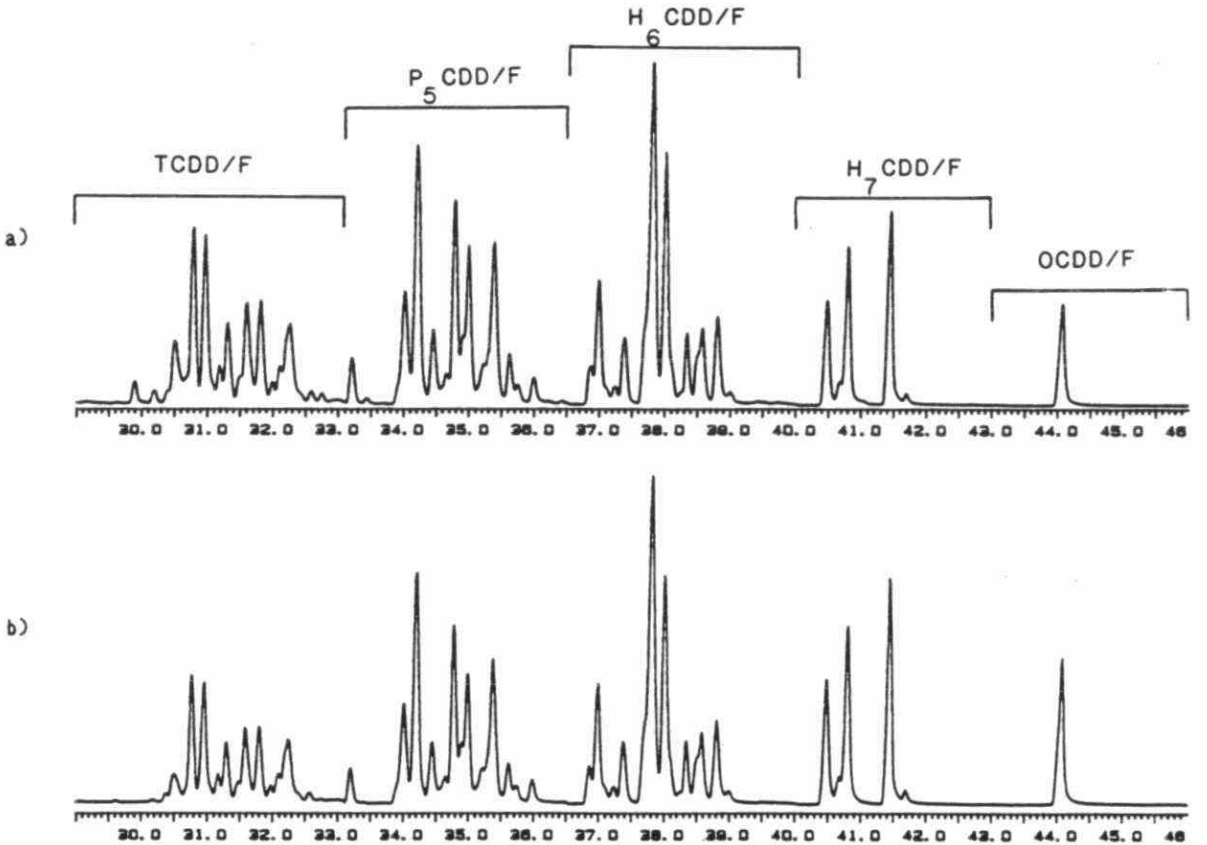

FIGURE 4: Total ion chromatograms obtained from GC/EIMS SIM analyses of a) flyash extract and b) flyash extract fractionated by HPLC (see table III for GC/MS conditions)

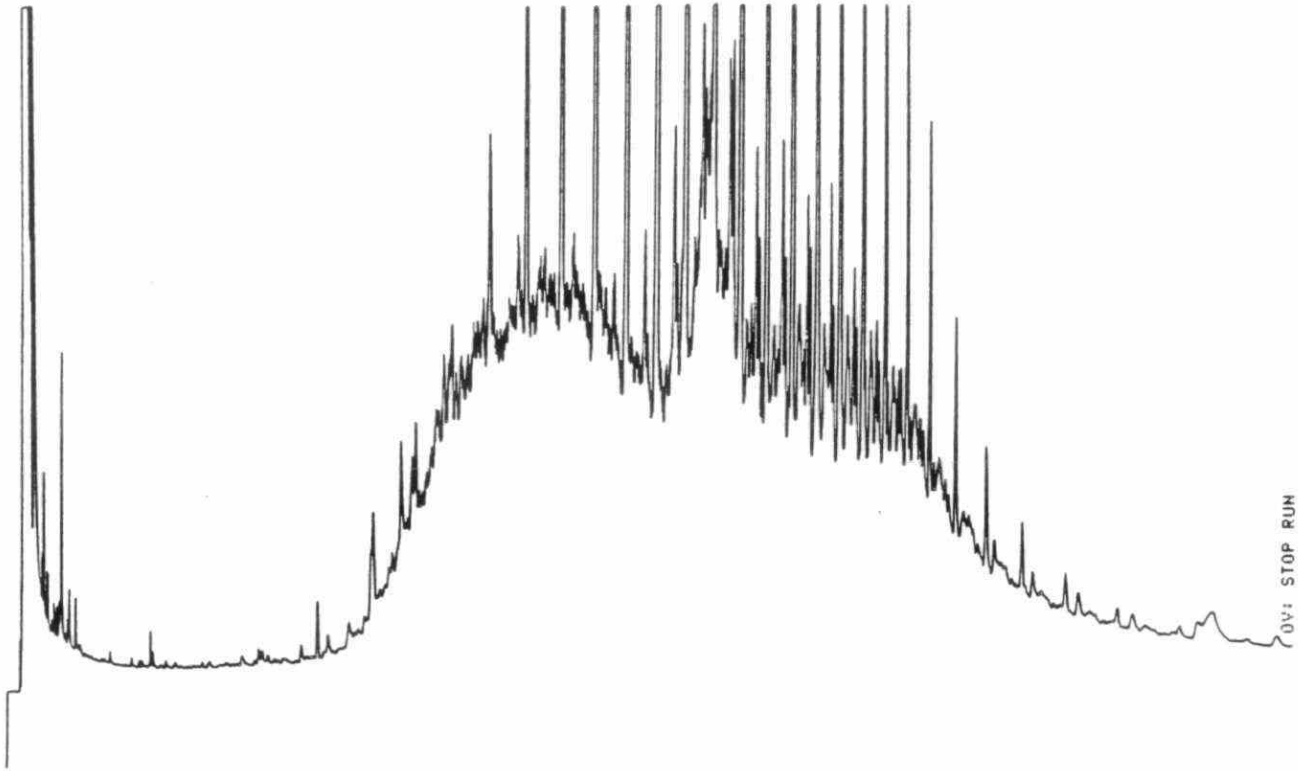

FIGURE 5: GC-FID chromatogram for HPLC fraction #1 of fish tissue extract (for GC conditions see table II)

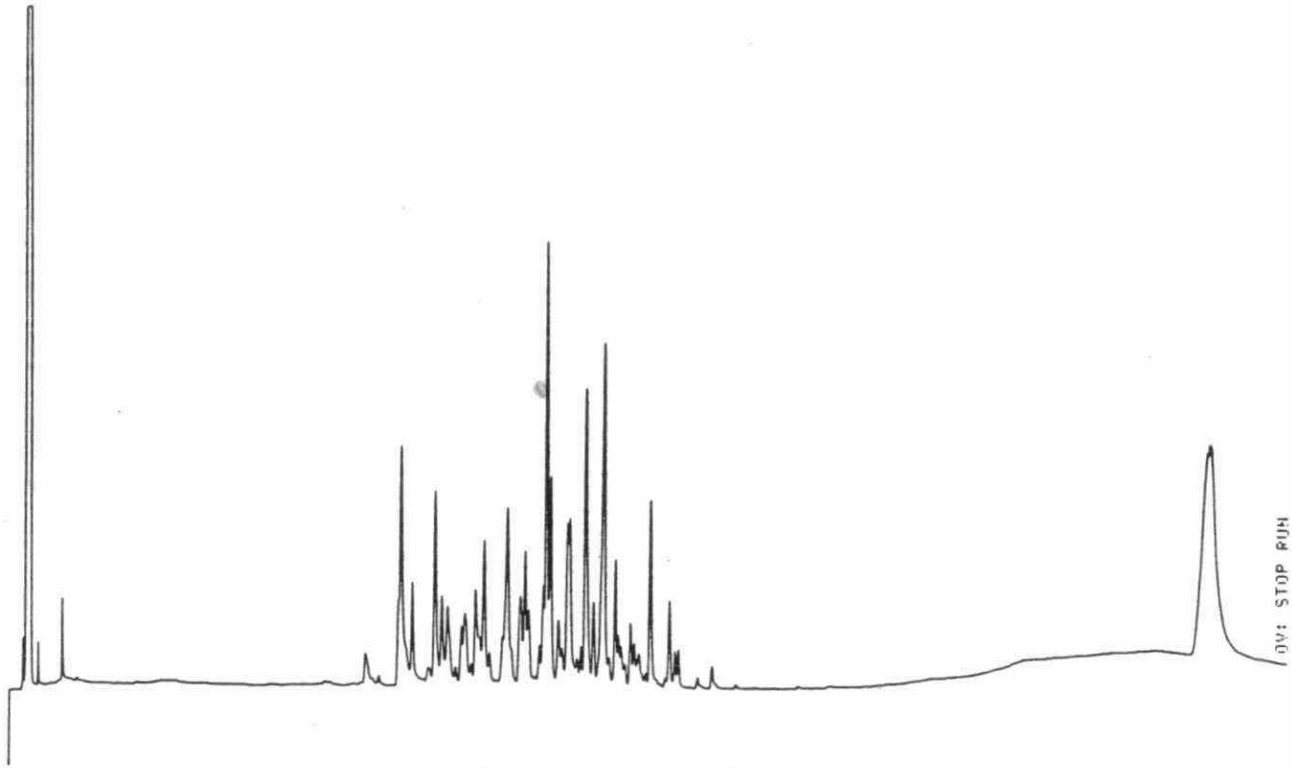

FIGURE 6: GC-ECD chromatogram for HPLC fraction #1 of fish tissue extract (for GC conditions see table II)

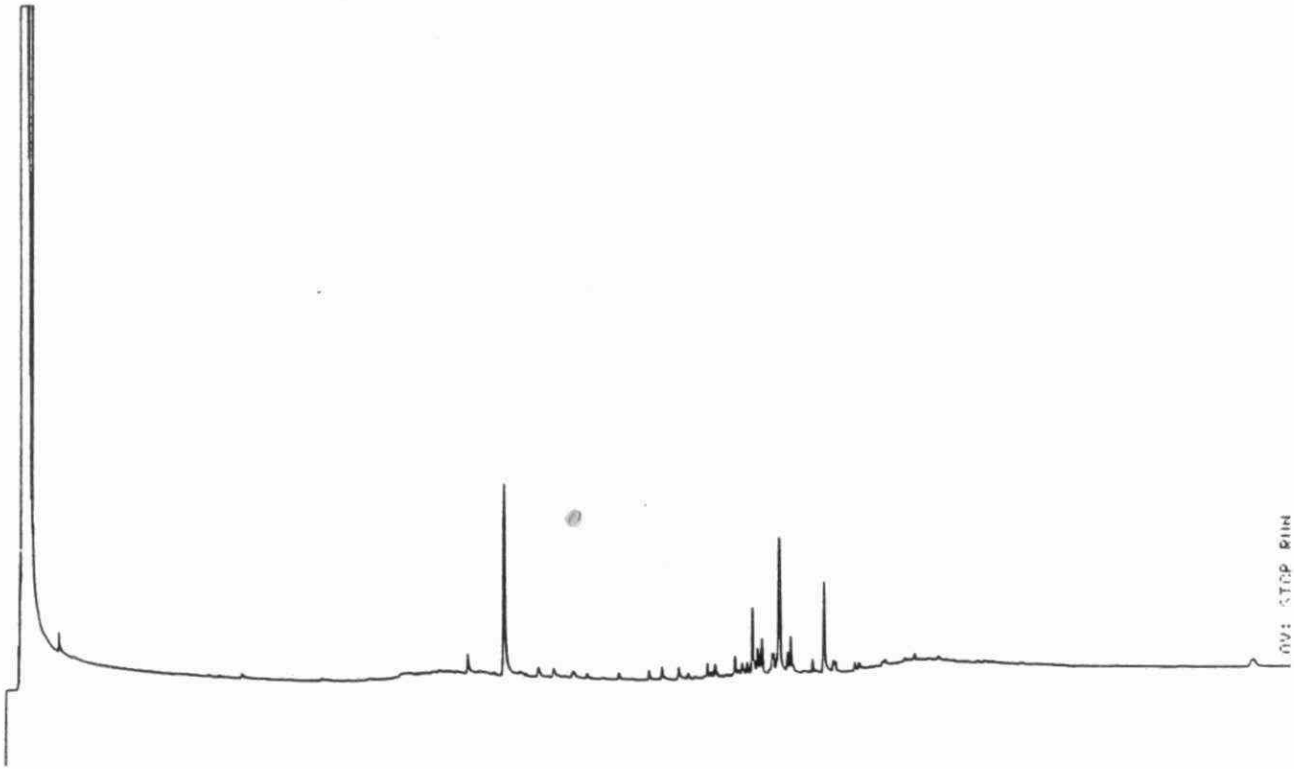

FIGURE 7: GC-FID chromatogram for HPLC fraction #2 of fish tissue extract (for GC conditions see table II)

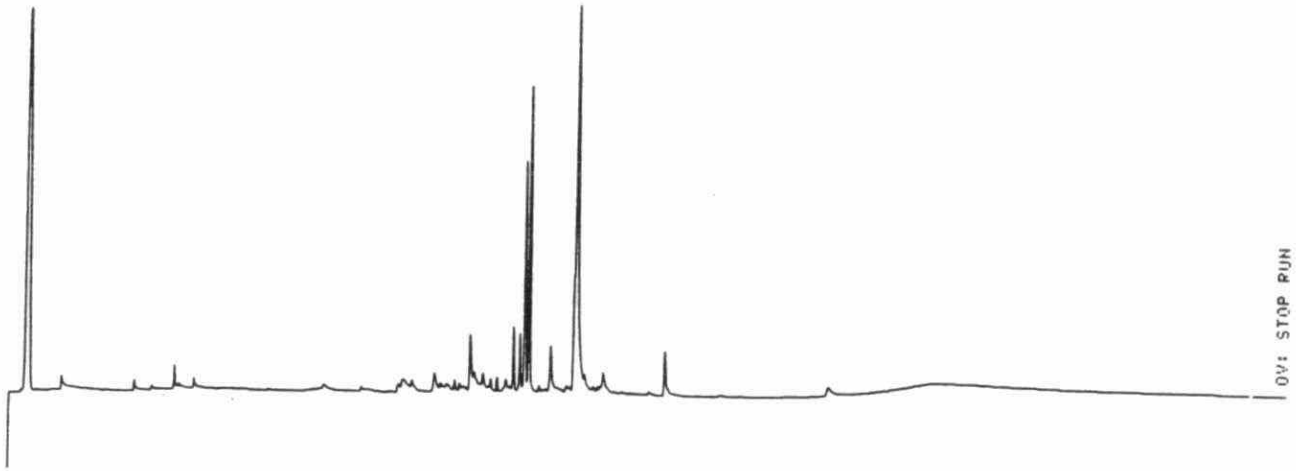

FIGURE 8: GC-ECD chromatogram for HPLC fraction #2 of fish tissue extract (for GC conditions see table II)

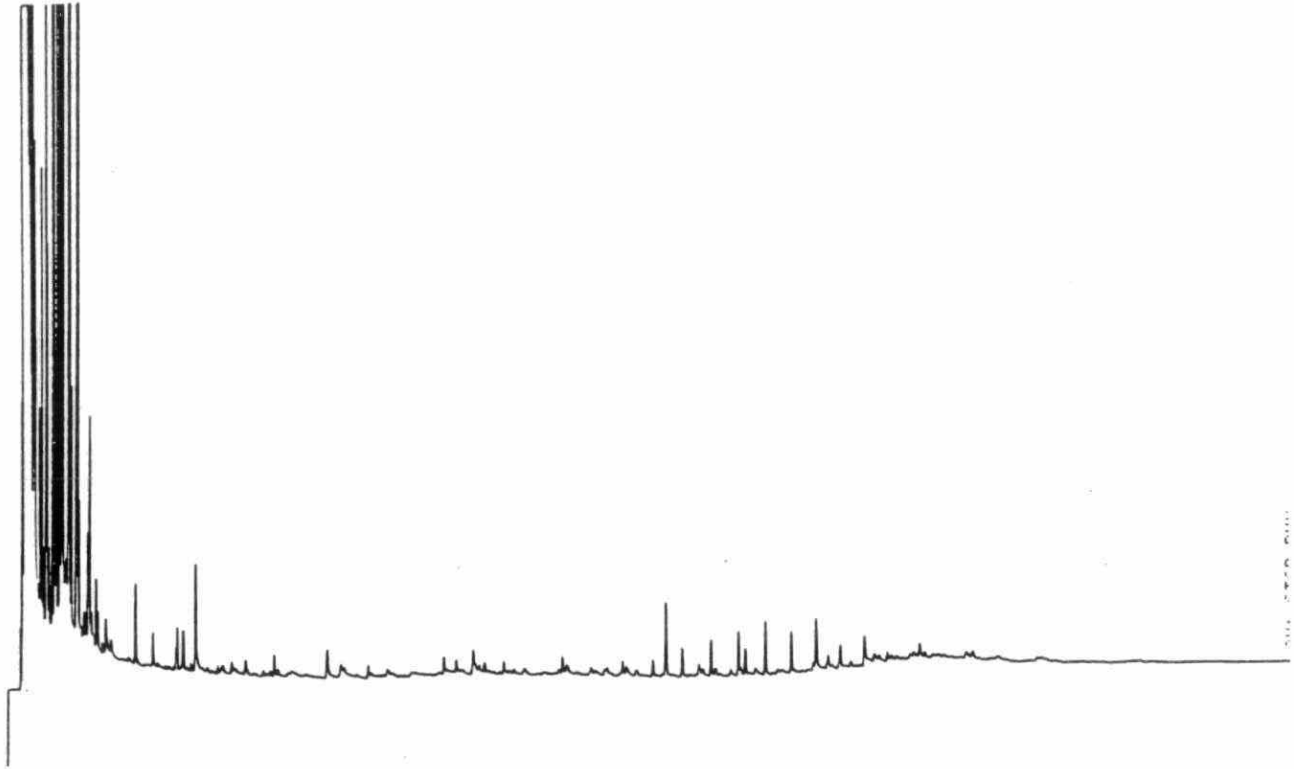

FIGURE 9: GC-FID chromatogram for HPLC fraction #3 of fish tissue extract (for GC conditions see table II)

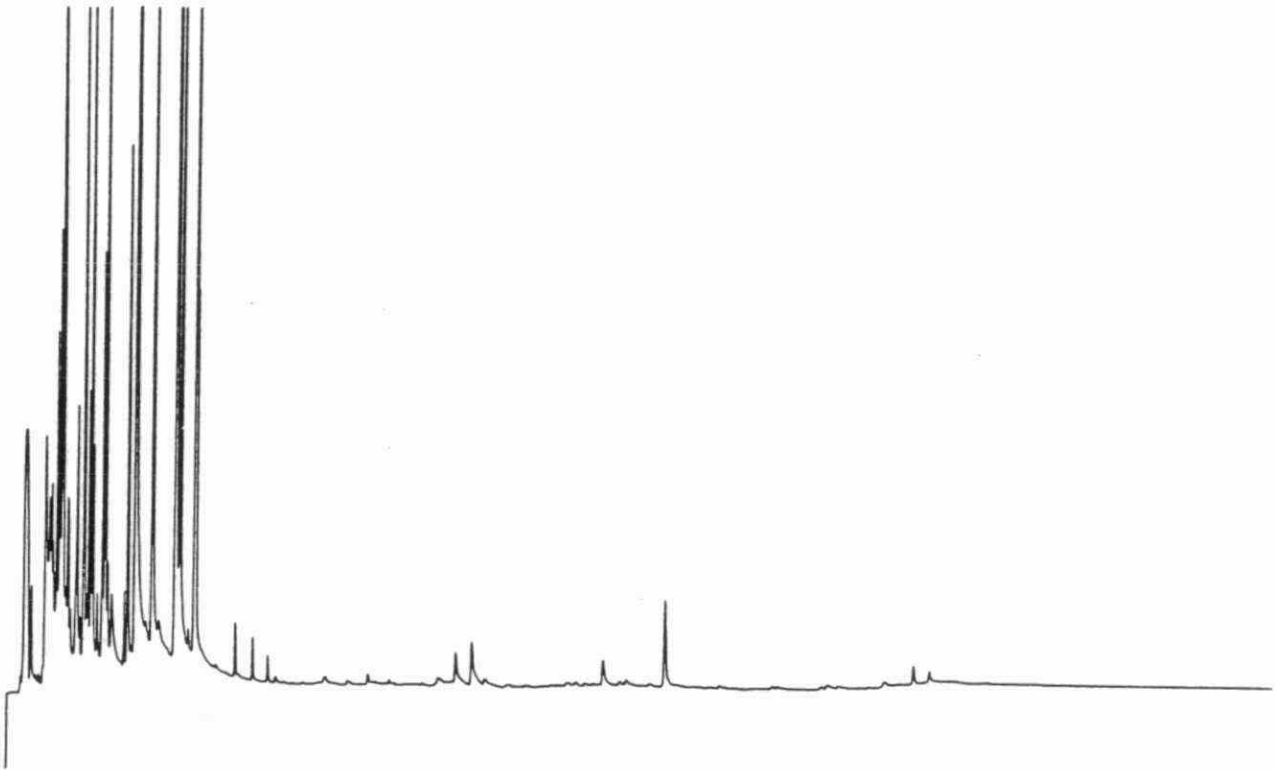

FIGURE 10: GC-ECD chromatogram for HPLC fraction #3 of fish tissue extract (for GC conditions see table II)

TABLE IV

COMPOUNDS IDENTIFIED IN FRACTION #1

Compound	MW	EI	NCI
dichlorobiphenyl	222	x	
pentachlorobiphenyl	324		x
pentachlorobiphenyl	324		x
hexachlorobiphenyl	358		x
tetrachlorobiphenyl	290		x
eicosane	282	x	
tetrachlorobiphenyl	290		x
hexachlorobiphenyl	358		x
hexachlorobiphenyl	358		x
pentachlorobiphenyl	324		x
heneicosane	296	x	
pentachlorobiphenyl	324		x
hexachlorobiphenyl	358		x
heptachlorobiphenyl	392		x
pentachlorobiphenyl	324		x
p,p'-DDE	316		x
pentachlorobiphenyl	324		x
hexachlorobiphenyl	358		x
hexachlorobiphenyl	358		x
docosane	310	x	
hexachlorobiphenyl	358		x
hexachlorobiphenyl	358		x
heptachlorobiphenyl	392		x
pentachlorobiphenyl	324		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
hexachlorobiphenyl	358		x
hexachlorobiphenyl	358		x
tricosane	328	x	
hexachlorobiphenyl	358		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
hexachlorobiphenyl	358		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
tetracosane	338	x	
hexachlorobiphenyl	358		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x
hexachlorobiphenyl	358		x
heptachlorobiphenyl	392		x
octachlorobiphenyl	426		x
heptachlorobiphenyl	392		x
heptachlorobiphenyl	392		x

TABLE IV
(cont'd)

COMPOUNDS IDENTIFIED IN FRACTION #1

Compound	MW	EI	NCI
pentacosane	352	x	
octachlorobiphenyl	426		x
octachlorobiphenyl	426		x
heptachlorobiphenyl	392		x
branched hexacosane	366	x	
octachlorobiphenyl	426		x
octachlorobiphenyl	426		x
hexacosane	366	x	
octachlorobiphenyl	426		x
branched heptacosane	380	x	
heptacosane	380	x	
octacosane	394	x	
branched nonacosane	408	x	
nonacosane	408	x	
branched triacontane	422	x	
triacontane	422	x	
hentriacontane	436	x	
dotriacontane	450	x	
tritriacontane	464	x	
tetratriacontane	478	x	
pentatriacontane	492	x	
hexatriacontane	506	x	

MW - molecular weight of compound

EI - identification based on EI mass spectrum

NCI - identification based on NCI mass spectrum

TABLE V

COMPOUNDS IDENTIFIED IN FRACTION #2

Compound	MW	EI	NCI
dichlorobiphenyl	222	x	x
heptachlorinated unknown	406		x
heptachlorinated unknown	406		x
heptachlorinated unknown	406		x
octachlorinated unknown	440		x
alkyl phenanthrene	234	x	
p,p'-DDD	318	x	x
octachlorinated unknown	440		x

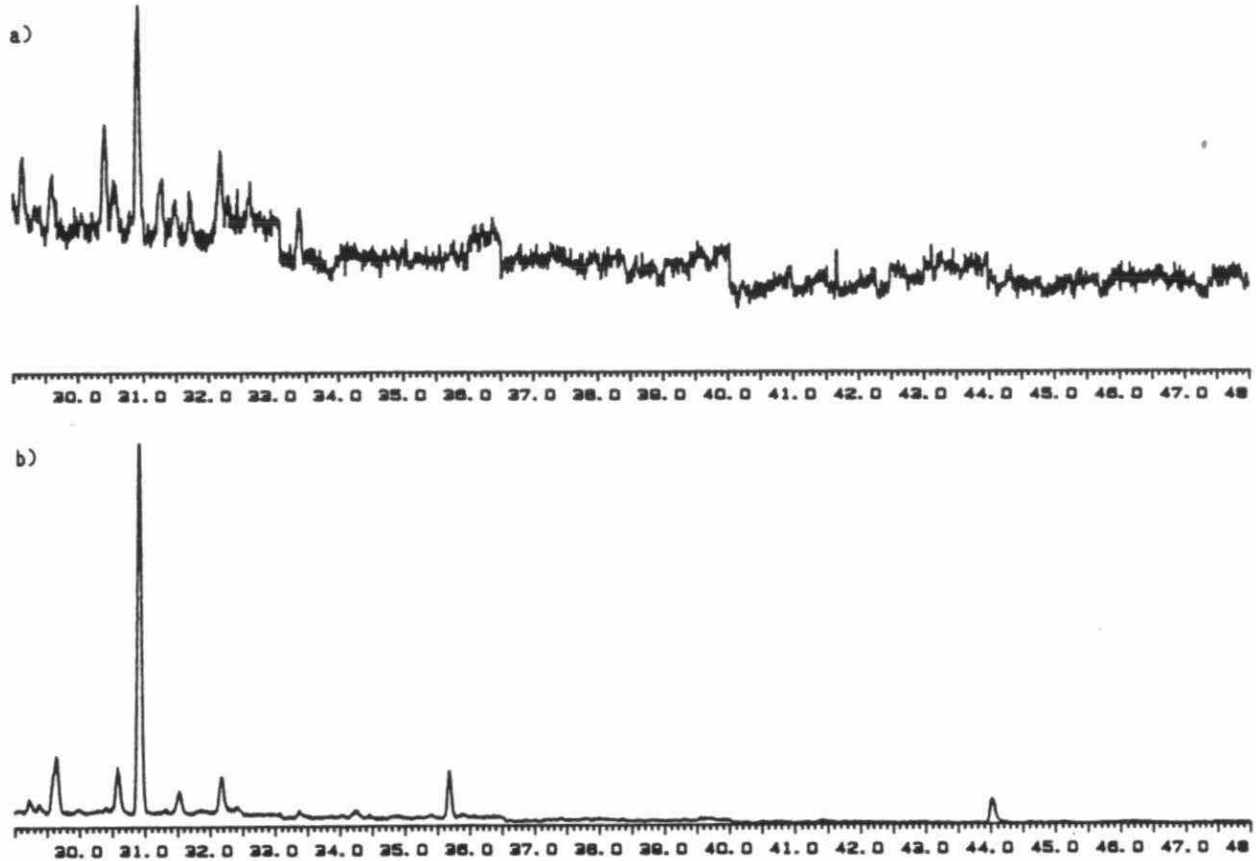

FIGURE 11: Total ion chromatograms obtained from a) GC/EIMS and b) GC/NCIMS SIM analyses of HPLC fraction #2 of fish tissue extract (for GC/MS conditions see table II-I)

predetermined criteria are satisfied. These criteria are outlined below.

1. The corresponding GC peak must elute within the correct retention window for a given congener group

2. Mass peaks corresponding to all three molecular ions monitored in the SIM analysis must be present

3. The ratio of the abundances of the mass peaks corresponding to the three molecular ions monitored must be within $\pm$ 10% of the theoretical ratios as determined by the natural abundances of ^{35}Cl and ^{37}Cl

4. The mass peak corresponding to the loss of COCl from the molecular ion species must be present

Under EI conditions there are peaks observed in the PCDD/PCDF retention windows however the ratios of the peak areas do not match the theoretical ratios of the intensities of the molecular ions. Therefore based on this fact alone, the peaks observed cannot be due to either dioxins or furans. Under NCI conditions the formation of an $[M-COCl]^-$ ion resulting from the loss of COCl is not observed. Therefore the criteria for identification of a component as a PCDD or PCDF is based upon the first three conditions. Several peaks are observed in the GC/NCIMS SIM profile (see figure 11). Closer examination of the data reveals that only the peak eluting at 44 minutes is a dioxin. Figure 12 shows the ion profiles corresponding to the three molecular ions expected for octachlorodioxin. The three criteria are satisfied and therefore the identity of this component is assigned as OCDD.

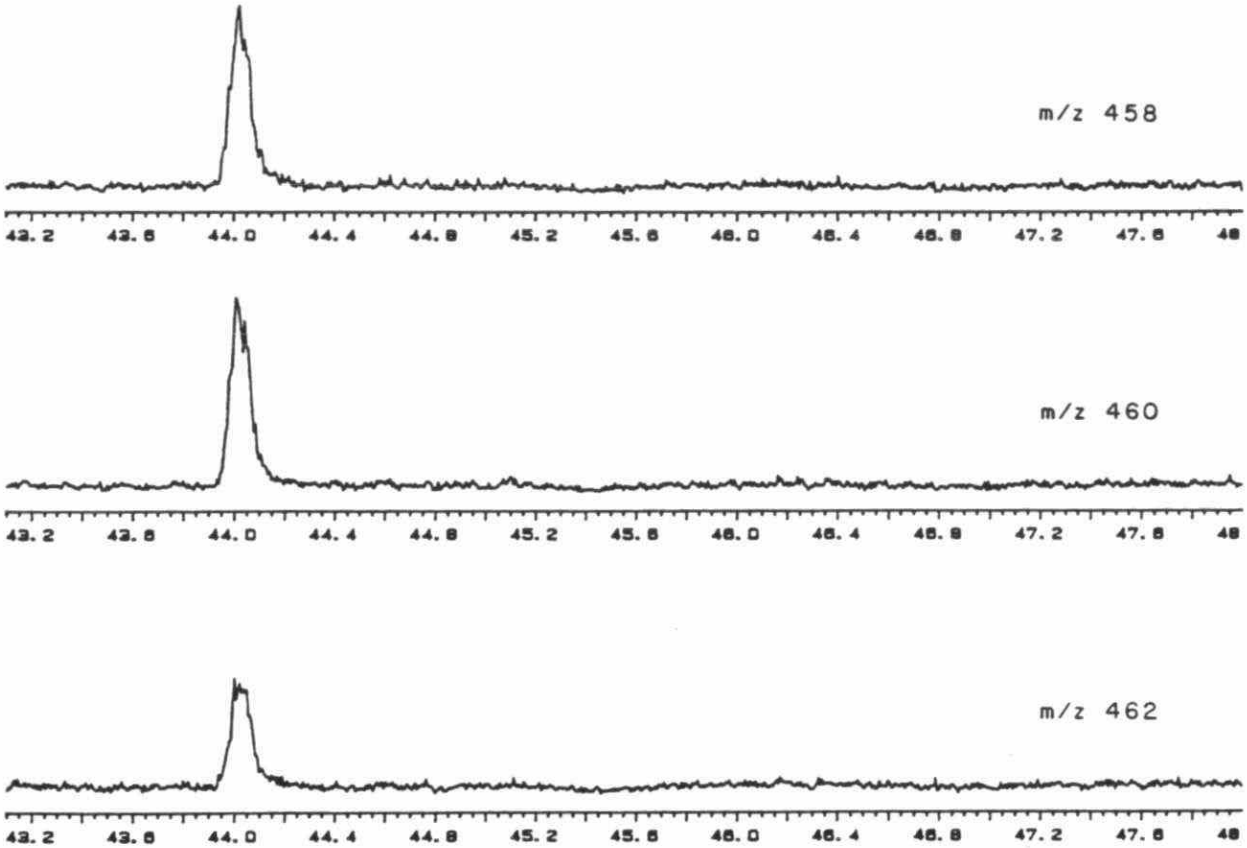

FIGURE 12: GC/NCIMS selected ion profiles of the masses corresponding to the molecular ions of octachlorodibenzo-p-dioxin

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"AUTOMATED WATER SAMPLER FOR DIOXIN DETECTION AT PPQ LEVELS", B. Hollebone, L. Brownlee and C. Shewchuk, Chemistry Department, Carleton University, Ottawa, Ontario; S. Suter and H. Tosine, Laboratory Services Branch, MOE; R. Hunsinger, B. Jobb and M. Uza, Water Resources Branch, MOE.

The Ministry of the Environment is responsible for a monitoring program for organic contaminants in drinking water. It is necessary to be able to detect and quantify accurately the levels of highly toxic classes of polychlorinated dibenzo-p-dioxins (PCDD's) and chlorinated dibenzofurans (PCDF's) in the environment. However, these compounds are present in trace amounts so it is necessary to analyze large volumes of water. The water is currently sampled using traditional grab sampling techniques and preconcentrated by the tedious process of liquid-liquid extraction. Public demand for more information on water quality has stretched the detection limits and sample handling capacity at the Laboratory Services Branch, Ministry of the Environment to the limits of the present technology. In order to fulfill future demands the Ministry of the Environment must lower the detection limit to 1.0 ppq while at the same time increasing sample load and maintaining high standards of Quality Assurance--Quality Control.

One approach to solving this problem is to move the preconcentration step from the laboratory to the field. Attempts have been made to perform the extraction directly in the field with APEL technology. However, current work with the macroreticular resin Amberlite XAD-2 has shown that PCDD and PCDF preconcentration utilizing such adsorbant materials is a reliable and compact alternative to liquid-liquid methodology (1). Resin columns can be prepared under laboratory conditions, sent to the field for sampling and then returned to the laboratory for elution and analysis. Automation of this process will standardize sampling and thus increase sample reliability, and minimize supervision requirements during operation. This process will eliminate the problem of transporting water samples or the handling of large solvent volumes in the field as only the columns must be transported. The sampling and preconcentration steps are combined, minimizing laboratory time and freeing technicians for other work.

Preliminary work on this technique in conjunction with National Health and Welfare and the Ministry of the Environment was directed at optimization of the XAD-2 resin technique. A semi-automated sampler functioned well for the preconcentration of treated water (2). However, MOE protocol requires sampling of both raw and treated waters. In raw water PCDD's and PCDF's in particular may be adsorbed on the inorganic or organic particulate. The XAD-2 resin in a column does not have the filtration capacity to remove particulate from the water and may not be able to compete with the particulate for adsorption of the organic contaminants. Therefore, an analytical filtration system was introduced into the sampler.

Object of Research

The object of this research was to develop an automated sampler capable of sampling reproducibly 100 liters of treated water or raw water with up to 100 NTU turbidity in duplicate for preconcentration of PCDD's and PCDF's at the ppq level. The sampler must be self contained with safety features carefully designed to eliminate any possiblity of exposure to PCDD's and PCDF's within a water treatment plant. Safety features include a feedback monitoring system that will automatically shutdown the sampler if there is any indication of mechanical failure, a water input mechanism designed to put a physical break between the water source and the sampler,and an activated charcoal filter at the output to eliminate any residual organics before exhausting sampled water.

The sampler must also have the analytical filtration capacity necessary to handle raw waters with high turbidity. Cartridge depth filters with a large surface area are commercially available for qualitative water filtration. Representative filters will be tested for their adaptability to analytical procedures. Tandem filtration and adsorption sampling techniques will determine the distribution of soluble and particulate-bound dioxins.

Research Approach

This project was developed in three phases; design and manufacture of the sampler , testing of cartridge filtration feasibility,and field testing of the completed sampler. The first two parts were completed concurrently. The sampler was designed and manufacutred in conjunction with Carleton University Science and Technology Center. A schematic of the sampler is given in figure 1. The water flows into the sampler via an air lock that provides a physical separation between the water supply and the sampler. A diaphragm pump feeds the water at a fixed flow rate through the filter/column assembly to a digital flow sensor. The flow sensor provides the flow rate feedback to the pump to maintain a constant input flow rate. The filters may partially block during the sampling process so to maintain constant flow, the input is designed to increase water pressure. An upper pressure limit of 60 psig is enforced by a pressure sensor. The pressure may be monitored visually by a pressure gauge. The flow rate may be monitored visually by a digital flow meter and a chart recorder provides a permanent record of sampling events. The filtered water passes through an activated charcoal filter before leaving the sampler. The entrance and exit to the sampler are each protected by a normally closed valve (NCV) which is operated by a batch controller. The batch controller is the center of the electrical control system. It is electrically connected to the NCV's, flow sensor and pressure switch, measures the total sample volume in liters and controls the operation of the system.

An optional spike assembly designed to improve QA-QC by dynamic spiking, will inject an analytical internal spike into the main water flow. A static mixer homogeneously mixes the spike and water sample. The spike assembly is connected to the batch controller by a microprocessor that will maintain a constant ratio between the main water flow and the spike injection. When not in use, the spike assembly is separated from the system by an NCV.

After the static mixer, the water flow is divided into two identical streams for duplicate sampling. Two manually adjusted ball-valve flow meters equalize the water flow. The sampler components are secured within an aluminum frame. Removeable aluminum side panels are sealed to retain any water spill within the sampler.

There are no cartridge filters commercially available that have been designed for analytical work. A market search for potential analytical cartridges was done. Suitable filters were extracted with toluene, dichloromethane or hexane to determine possible analytical interferences. The cartridges were also tested to determine their capacity for handling raw waters with high turbidity. The recovery of PCDD's from cartridge filters will also be tested. In the third phase, the performance of the sampler will be tested under field conditions.

Main Findings to Date

We have found that most available cartridge filters are unsuited for analytical work. We require a combination of a depth filter to handle the high particle content of raw water with a membrane filter for smaller particles in the 0.45 micron range. Sampler filters were tested by soxhlet extraction of the individual materials with hexane, toluene, and dichloromethane. All polypropylene materials reacted with hexane and toluene. Urethane endcaps and glue, used to seal endcaps to filter, reacted with all solvents, especially dichloromethane. To date, the most suitable cartridge is a glass fiber depth filter combined with a cellulose acetate membrane filter that is contained within a completely polypropylene support and is heat sealed to the filter. The most suitable extraction solvent is dichloromethane. The filter companies have been very cooperative and several have expressed an interest in manufacturing a more suitable cartridge when needed.

Preliminary spiking studies using a combined filter/column system show that most PCDD spike is located on the filters suggesting that these contaminants are either particulate-bound or directly adsorbed onto the filter. This is very useful information because it suggests that sampling for dioxin may be combined with sampling procedures for water-soluble pollutants.

Major Problems

There have been three major problems to overcome within this study. The first was the limitations of the mechanical components. Most commercially available components were designed for industrial applications that require a larger capacity so that in this situation most of the components are operating at their lower limits. A metering pump capable of injecting 0.2-1.0 mL per minute of spike with digital feedback control was not commercially available and is being manufactured by the Science and Technology Center. All components must be made with stainless steel or teflon as any organic components may contaminate the analytical process. One major limitation of the sampler is the adaption to high turbidity raw water. In this sampler high precision instruments are necessary for analytical reliability, however, in many components raw water particulate interferes with this precision.

The second problem was the inability to find a suitable commercially available analytical cartridge filter and has been discussed earlier. The third problem was the need to find a standard raw water source to test the capacity of the filters for handling high turbidities. Natural sources vary with weather and season. We created an artificial raw water from standard clay and organic sources. This will allow us to compare the behaviour of different filter types at several turbidities without the variability of natural waters.

Future Potential of Sampler

The main thrust of this project was to design and construct an automated sampler to a state of reliable performance equivalent to analysis by grab sampling. This will be completed within the limitations of the commercially available cartridge filters. Further work on the design, optimization and extraction of custom filters may be necessary to reach the full potential of the preconcentration system. Once the system protocol has been optimized for easy handling and reliable analysis, this preconcentration method could be implemented on a routine basis across the province.

This sampler also has the potential to be used to monitor environmental contaminants other than dioxins. The environmentally acceptable levels of many other organic and metal contaminants have been set below the detection limits of current techniques. With the extensive column technology now commercially available this preconcentration system could be optimized for many other analytical procedures.

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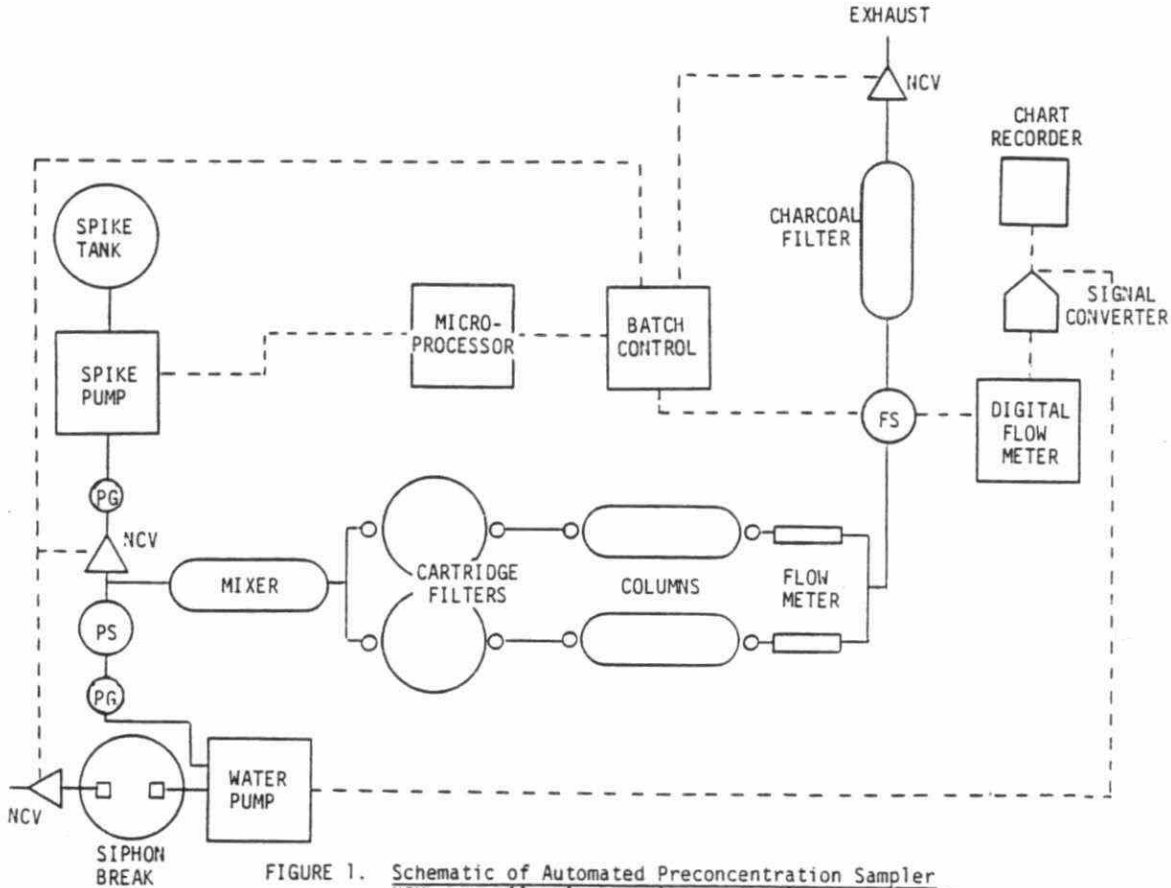

FIGURE 1. Schematic of Automated Preconcentration Sampler
NCV- normally closed valve; FS- flow sensor; PS- pressure sensor; PG- pressure gauge; O- 2-way valve; _____- plumbing connections; _ _ _- electrical connections.

HPLC FRACTIONATION PROCEDURES FOR THE ISOMER SPECIFIC ANALYSIS OF PCDD AND PCDF IN ENVIRONMENTAL SAMPLES

Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs) are two groups of compounds which have recently become the subject of a considerable attention because of their toxicities and their persistence in the environment. Each group actually consists of a large number of different isomers arising from varying degrees of chlorination and the different positional sites which can be occupied on the aromatic rings. Figure 1 shows the numbering system used in the naming of dioxins and furans. The number of isomers possible for each congener group are given in table I.

The toxicity of a dioxin or furan varies greatly depending upon the number and position of the attached chlorine atoms. The most toxic isomers are the tetra-, penta-, and hexachlorinated species in which a chlorine atom occupies each of the 2, 3, 7, and 8 positions (1). These isomers are listed in table II.

The differences in toxicity exhibited by the individual compounds makes it desirable to perform isomer specific analyses on environmental samples. The large number of possible isomers greatly complicates the analytical methods required to achieve the isomer specific determination of PCDDs and PCDFs.

Mass spectrometry is commonly used for the analysis of dioxins and furans because of its sensitivity and specificity. It is impossible to distinguish between positional isomers (for example, two different TCDDs) based solely on the appearance of their electron impact mass spectra. Therefore separation of the

isomers, usually achieved by chromatographic techniques, must be done prior to mass spectral analysis. A directly linked gas chromatograph/mass spectrometer system would appear to be the answer. However even with the recent advances in technology for chromatographic columns, there is no single GC column capable of separating all PCDDs or PCDFs.

Buser and Rappe reported the preparation of all the tetra-, penta-, and hexachlorodioxins (1). Using a 55 metre Silar 10c (cyanosiloxane) glass capillary GC column all of the toxic PCDDs listed in table II were separated from the remaining dioxins. It should be noted that 2,3,7,8-TCDD (the most toxic dioxin) could not be completely resolved from 1,4,7,8-TCDD and two of the most toxic hexachlorodioxins, 1,2,3,6,7,8-H_6CDD and 1,2,3,4,7,8-H_6CDD, elute very closely together. Although the five most toxic dioxin can be reasonably well separated from the remaining PCDDs many of the others coelute.

In an earlier study Buser and Rappe reported the synthesis and high resolution GC separation of the 22 TCDD isomers (2). Using 3 HRGC columns (55 metre Silar 10c, 50 metre OV-17, and 50 metre OV-101), 14 isomers could be unambiguously isolated using a combination of separate injections on the different columns. No single column was capable of separating more than 11 different isomers. Clearly a more rigorous separation procedure is required.

Nestrick and co-workers synthesized the 22 TCDD isomers and used high performance liquid chromatogaphy (HPLC) to fractionate mixtures of the dioxins prior to GC/MS analysis (3). A two-step

HPLC fractionation procedure was used to isolate all 22 isomers. In the first HPLC separation, two C_{18} reverse phase columns (6.2 x 250 mm) connected in series and isocratic methanol were used. Each fraction collected from the reverse phase HPLC (RP-HPLC) separation was further fractionated on normal phase HPLC (NP-HPLC). Two silica columns (6.2 x 250 mm) in series were used to achieve the separation of the TCDDs in the various RP-HPLC fractions.

This method of separating the TCDD isomers was modified by another group of workers (4). They found that recoveries were poor for sample loads of less than 10 ng per component. The NP-HPLC adsorbent activity and the mobile phase solvent system were slightly modified resulting in a reported increase of recovery from 20 to 80 percent.

Although some success has been reported in isomer specific dioxin analysis, little work has been reported on isomer specific determination of chlorinated dibenzofurans. For very complex samples it is not sufficient to isolate the isomers of interest from the remaining PCDDs and PCDFs. There are numerous other interferences many of which are present at levels much higher than the dioxins or furans. In our research we have found that NP-HPLC using an alumina column provides the separation of PCDDs and PCDFs from many interferences. The usefulness of this column in isolating dioxin and furan isomers has never been reported in the literature. The investigations performed in our laboratory require further testing at the time of the preparation of this manuscript and the results will be presented at the conference

itself.

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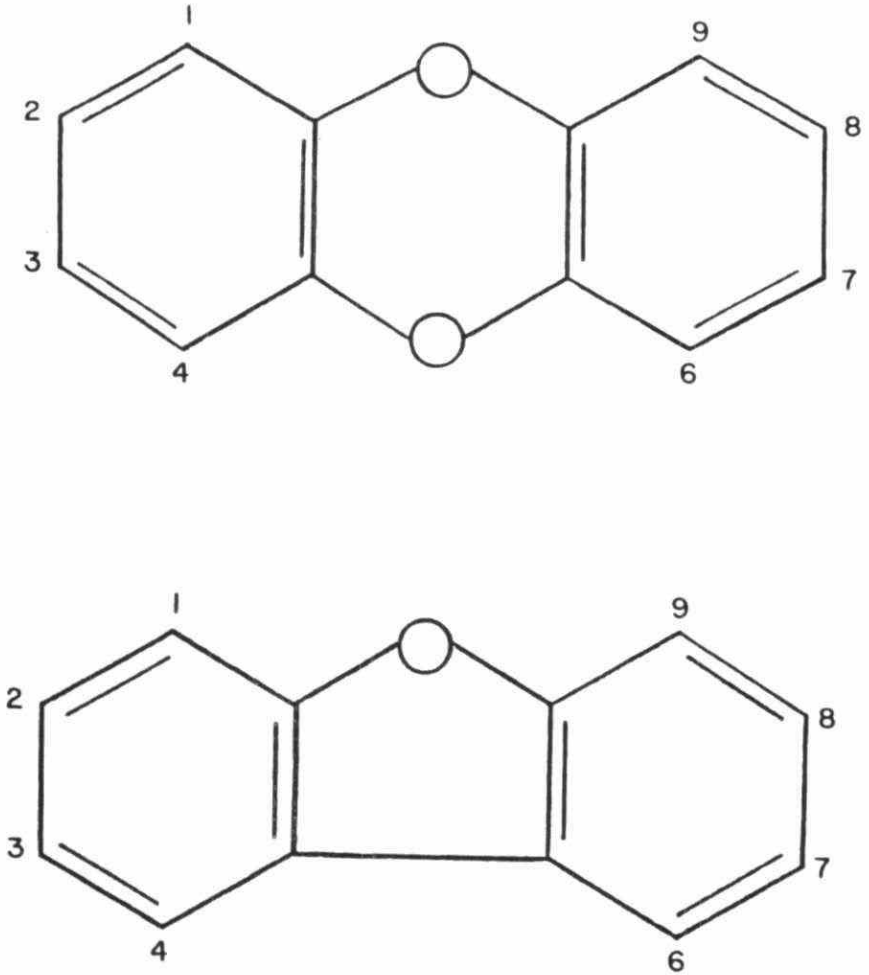

FIGURE 1: Numbering system for naming PCDDs and PCDFs

TABLE I

DISTRIBUTION OF DIOXIN AND FURAN ISOMERS

# OF CHLORINE ATOMS	# OF ISOMERS	
	PCDDs	PCDFs
1	2	4
2	10	16
3	14	28
4	22	38
5	14	28
6	10	16
7	2	4
8	1	1
TOTAL	75	135

TABLE II

MOST TOXIC PCDDs AND PCDFs

PCDDs:	2,3,7,8-TCDD
	1,2,3,7,8-P_5CDD
	1,2,3,6,7,8-H_6CDD
	1,2,3,7,8,9-H_6CDD
	1,2,3,4,7,8-H_6CDD
PCDFs:	2,3,7,8-TCDF
	1,2,3,7,8-P_5CDF
	2,3,4,7,8-P_5CDF
	1,2,3,4,7,8-H_6CDF
	1,2,3,6,7,8-H_6CDF
	1,2,3,7,8,9-H_6CDF
	2,3,4,6,7,8-H_6CDF

DEVELOPMENT OF A ROBOTIC SYSTEM FOR THE ROUTINE ANALYSIS OF PESTICIDES IN BIOTA BY ACID DIGESTION AND SOLVENT EXTRACTION SOLVENT EXTRACTION

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INTRODUCTION

The increased public awareness of the presence of persistent toxic chemicals in the food chain has led to an increase in the number of analyses that must be performed annually. To meet this requirement, laboratories have to become more automated to deal with large number of repetitive samples with high priority. Laboratory automation in the past decade has primarily been focused at the sample analysis and data processing areas. Automated sample preparation and processing have not been addressed until recently. Since the sample preparation steps are usually time consuming, repetitive and prone to error, a robotic system in lieu of human operation is thus a logical choice.

This paper presents an overview on the development of a robotic workstation for the preparation of fish samples using acid digestion method. The system being used to implement this procedure is the Zymate II Laboratory Automation System, manufactured by Zymark Corporation of Hopkington, Mass.

The system design objectives are as follows:

i. Improved precision and accuracy of results.

ii. Cost savings in materials such as solvents.

iii. Reduced operator exposure to hazardous chemicals.

iv. Improved data reduction and reporting.

v. User friendliness.

The development of this system included the acquisition and modification of the hardware required by the analytical method, the establishment of the robotic procedures based on precise specifications of a manual method, the design and testing of software, and validation of the completed system.

SYSTEM HARDWARE

After evaluation of the manual method and the availability of robotic

workstations, the following robotic components were selected:

i. Robot arm and Controller

ii. Power and Event Controller

iii. Robotic Hands (General Purpose, Blank and Cannular)

iv. Master Laboratory Station and Remote Pipetting Kit

v. Electronic Balance

vi. Sample Conditioning Station (Linear Shaker)

vii. Capping Station

viii. Remote Computer Interface

ix. Centrifugation Station

x. High Volume Dispensing Kit

xi. Drying/Neutralization (Prep-Sep) Station

xii. 10-ml Pipette Kit

xiii. Waste/Rinse Station

xiv. Cannular Wash Station

xv. Assorted Racks for Centrifuge Tubes

xvi. Purge Kit

xvii. Printer

Contract specifications also required that all sample attributes be stored on a remote PC and that a report be generated for each sample. To fulfill this requirement a microcomputer (IBM compatible) with 20 MB hard-drive, floppy disc drive and two serial ports were obtained and interfaced to the system.

Software used in the development consisted of Zymark's EasyLab language for robot controller programs and GW-BASIC running under DOS on the PC. Selection of the Basic language was influenced by the fact that most users are familiar with it, and since it is an interpreted rather than compiled language, it will

consequently speed software development.

DESCRIPTION OF MAJOR WORKSTATION COMPONENTS

a) Robot Module

The "robot" itself consists of an "arm" secured to a benchtop by a pedestal. This allows three degrees of freedom of motion. The arm is capable of holding a variety of "hands", which rotate about a wrist connection, giving one extra degree of freedom.

The range of motion in each direction is:

(a) Vertical : 0-34 cm
(b) Reach : 0-32 cm
(c) Rotary : $0\text{-}376^0$

The arm's locomotive power is provided by a wire pulley system, driven by DC servomotors. Directional control is provided by programmed commands. Initial calibration of the arm at the factory provides a frame of reference, so the arm "knows" it's current position. To go to a new position, the controller compares the current position to the desired position. This generates an error signal, which drives the servo's in such a way as to minimize the error signal.

There are four different types of hands available to this system:

(a) General Purpose Hand
(b) Syringe Hand
(c) Dual Function
(d) Blank Hand

In this application, the General Purpose Hand, the Blank Hand and the Cannular Hand are used. The General Purpose Hand consists of a Teflon block housing a servomotor and has two "fingers" (grippers) attached. This hand is used for manipulation of glassware. The joint of the hand and arm forms a "wrist" capable of rotation form -5 to 365^0. In addition, the force with which the fingers grip an object can be adjusted from 0 to 200 units.

The arm and hand assemblies can also receive feedback from the external world in each of three axis:

ie: Vertical Force
Reach Force
Rotary Force

as well as Grip Force in the hand. This allows the arm to "sense" possible error conditions while performing operations on the bench.

The arm also allows different speed settings for each axis and can sense a collision, in which case it stops, and can only be restarted by the operator intervention.

b) Master Laboratory Station and Remote Pipetting Kit

(i) The Master Laboratory Station

This station consists of a housing containing three 10ml syringes, valves to control fluid flow, stepper motors to drive the plungers, and a dispenser nozzle mounted on a post.

The syringes can be programmed either independantly or jointly, to dispense/fill from a reservoir. Plunger speed can also vary to compensate for fluids of different viscosities.

The Dispenser nozzle contains separate outlets to avoid cross contamination of liquids. The syringes are programmed by setting the volume, speed and flow conditions necessary in a given operation.

Accuracy and precision for this station are quoted at 0.5% and 0.2% respectively, where volumes are greater than 20% of total syringe volume.

(ii) Remote Pipetting Kit

The remote Pipetting Kit allows pipetting/dispensing to vessels at fixed positions on the benchtop. It consists of a Blank Hand with a pipet-tip holder and pneumatic shucker, the parking station for this

hand, and tubing for attachment to external compressed air, the MLS, solenoid operated valve, and a pipet-tip rack.

After attachment of this hand, programming is the same as for programming of any other robot moves. The pipet-shucking operation is driven from the Power and Event Controller, where actuation is by switch closure.

c) **Power and Event Controller**

The Power and Event Controller permits programmed operation of external devices. It provides the options listed in the manufacturer's documentation.

In this application, it is used to discard pipet tips and operate vacuum for the Rinse/Waste station. The large number of control functions will permit future expansion to the system, should it be required.

d) **Printer Module**

The system permits operation of a printer to provide hard copies of data and programs. It can be programmed to be either enabled or disabled and provides essentially a line by line screen dump.

e) **Balance Module**

The balance used in this application is a Mettler PE200. The module consists of the Balance Interface Card, a 5-pin to 32-pin cable and a sample tube holder. The balance is capable of sending weight results to the Controller via the balance interface card, which is compatible with Mettler's CL interface and capable of receiving commands from the

controller. It is a menu-driven progammable unit, where baud rate, parity and data transfer Mode may be set and stored. In addition, dampening functions may be set to compensate for vibration and time-averaged readings are also possible.

Operation is controlled by the Zymark controller, where weight and tare commands are defined. Results are stored in arrays for use in calculation of masses.

f) **Capping Station**

The Capping Station consists of the capper (an enclosed motor with a set of jaws on the top surface), and a cap parking station, where bottle caps are temporarily held.

The Capper's operation must be carefully coordinated with that of the arm. During a cap operation, a cap is retrieved from the parking station and lowered onto the tube while the capper rotates. Similarly, during an uncap operation, the tube is placed in the capper, the jaw grips the cap and the capper rotates in the opposite direction while the arm is incrementally raised.

The station can be programmed for capping torque and number of turns required to cap or uncap. Error routines are incorporated into the software to detect most common types of errors i.e. grip slipping etc.

g) **Drying/Neutralization Station**

This station consists of a prep-sep dispenser and a prep-sep workstation. It is run from the PEC, where a switch closure will result in pressure being applied to a prep-sep containing a solvent aliquot. The prep-seps contain a layer of sodium bicarbonate (0.5g)

and a layer of anhydrous sodium sulfate (0.5g). The pressure forces the solvent through the prep-sep cartridge, where the aliquot is dried and neutralized.

This workstation will save the expense of a powder pouring station and its associated equipment as well as bench space and time during the processing.

h) **Sample Conditioning Station**

The Sample Conditioning Station consists of a linear shaker and two vertical, five position rack. The station is used for the agitation of samples during digestion and extraction, where the arm will load the tubes in a horizontal position.

The shaker is capable of speeds from 1 - 100 units and always stops at the same position, to faciliate access by the robot arm during insertion/removal from the rack.

I) **Waste/Rinse Station**

The Waste/Rinse Station will be used to aspirate the fish/acid residue and unused solvent to a waste holding tank. It is run from the PEC, where switch closure results in vacuum being applied.

The Rinse part of this station consists of a Hi-Volume Dispensing Kit, which contains a high-speed peristatic pump, tubing, valving, post and dispenser nozzles. When switched on, the tube will be washed out with water, supplied by the pump.

OUTLINE OF SYSTEM CONTROLLER

a) Hardware

The Zymate II System employes several 16-bit chips. The main CPU is the Intel 8088, with an 8087 used as a math co-processor (the Fast Math upgrade from the Zymate I). Total memory is 64K, with the operating system occupying 4K and leaving 60K for user applications. RAM in this system is non-volatile and battery-supported.

The Zymark System is very flexible due to the modular concept. In this design, each module (ie Capper, MLS) has it's own module card, where it's intelligence is contained on ROM chips. The module card is linked to it's module by a control cable, and to the controller by module support boards. Each module support board can hold up to five module cards, and there is room for five module support boards, hence, we may have up to 25 modules in a given application.

The System may be interfaced to an external computer by use of the Computer Interface Module. This module provides an RS232/423, ASCII, serial communications link between the controller and an external, more powerful laboratory computer. The interface parameters that may be set are:

(i) Baud Rate : Up to 1200 Baud
(ii) Character Length : 7 or 8 bits/character
(iii) Stop Bits : one or two

This will allow the Computer Interface to communicate to almost any PC we choose to use, providing it has a communication board and uses the same RS 232 standard interface.

b) Software

The Zymate II System provides a menu-driven system for operator convenience.

Upon initialization of the system, the Controller polls the modules plugged into the module support boards, and configures the system accordingly. The initial menu is displayed, and gives one five choices:

1. Run Program: allows one to execute an existing EasyLab Program.

2. Edit Program: allows one to edit or create programs. This is a line-editor, sufficient for most purposes.

3. Bench and Module Setup: this selection displays the modules currently configured in the system. From this menu, we may enter a control "page" which gives direct control over that particular module. Each module has a control page where defaults are set or changed. An example is the computer interface, where the baud rate, parity and stop bits may be set, depending on the characteristics of the external computer.

4. Systems Management: displays the status of the RAM batteries, amount of dictionary space left, and allows one to specify an autostart program.

5. Direct Control: allows the execution of programs and commands directly.

The Zymate II allows programs to be written in it's language called EasyLab. This is an interpreted language and has a flavor characteristic of BASIC. For example, the following constructs are allowed:

(i) Arrays: numeric, single dimension only.

(ii) DO Loops

(iii) GOTO

(iv) IF conditions THEN statements : but no ELSE construct.

(v) Real Math: 6 Significant figures.

(vi) Standard Mathematical Functions : SIN, COS, TAN, SQRT and LOG

EasyLab software allows any user-defined variables to be globally available to any sub-routine called from within any program. The maximum number of levels of calls is limited to 7. Text processing is very limited,

as there is only one type of PRINT statement.

To overcome the programming limitations of Easy Lab and the Controller, an external computer was linked to the controller via the computer interface. With this configuration, a much more "intelligent" system is designed, where the external computer can be executing a more complex program, and simply pass the appropriate commands to the controller. Likewise, the controller can pass information back to the computer, where decisions will be made.

The choice of a high-level language running on the external computer is essentially wide open, but QUICK-BASIC, a compiled version of BASIC, was recommened.

CHANGE TO MANUAL PROCEDURE

In order to convert the manual extraction method to a robotic procedure the following modifications were made:

1. The original manual method requires extractions to be carried out in 60 ml centrifuge tubes. Since the capacity of the centrifuge module in the robotic system is for 50 ml tubes, all volumes were adjusted accordingly.

2. Robotic pipetting procedures were performed gravimetrically.

3. In the manual procedure, extract neutralization and drying steps were performed in Erlenmeyer flasks. To implement this step in the same manner would require too much bench space and additional glassware to be manipulated by the arm. In addition, the dispensing of powders are sufficiently time-consuming as to reduce sample throughput. Our solution was to proceed with disposable columns packed with sodium bicarbonate and anhydrous sodium sulfate, which required only one additional workstation.

4. The inclusion of a centrifuge module to break up emulsions and settle undigested fibrous material enabled better separation of phases.

5. The manual procedure required the final extract to be volumetrically diluted to 100.00 mls. in which a one-gram equivalent (or 1/5 of total volume) was removed for cleanup. Again, table space does not permit the required number of flasks. Instead, the total volume of extract was computed and a calculated volume was pipetted volumetrically.

DESCRIPTIONS OF ROBOTIC PROCEDURE

The final implementation of the manual procedure to a robotic procedure resulted in the following robotic method. This method is described as a linear version. Due to the sample throughput requirements, this linear procedure was serialized i.e. several processes and different samples are being processed concurrently on the table at the same time.

1. The initial step consists of taring a rack of 24 capped, empty centrifuge tubes, which will be loaded with samples. This step is performed independantly of the main run sequence. All weights are stored in an array.

2. After taring, the operator removes the rack of tubes and loads each with 5.0 $\pm$ 0.1 g of wet fish tissue. The tolerance of the weight must be adhered to, in order to ensure the centrifuge remains balanced. The loaded rack is replaced on the table and the run is initiated.

3. Operator enters run date, report header information, number of samples to be run, and sample attributes (sample code and type). A program is executed for all tubes where each tube is removed from the rack, loaded into the balance. The weight is stored in a data file and the tube is then unloaded from the balance and returned to the rack. This procedure is repeated for all tubes.

4. A sample tube is taken from the rack, and weighed to obtain actual sample weight.

5. When finished, the operator starts a digestion run. This consists of the following robotic steps:
   a) The loaded tube is removed from the rack.
   b) It is then weighed again on the balance
   c) A syringe on the Master Lab Station is prefilled with conc. HCl.
   d) The tube is uncapped and the cap parked.
   e) A 20 ml portion of acid is added to the sample.
   f) The tube is then capped.
   g) The tube is weighed.
   h) The tube which now contains the sample and acid is returned to the rack.
   i) This process is repeated sequentially for each tube.

6. At the meantime, samples are allowed to digest overnight. When the samples are digested, an extraction routine which consists of the following steps is executed.
   a) The tube containing the digested sample is removed from the rack.

b) The arm changes it's grip on the tube by loading it into the capper and shifting it's grip so it grasps the tube by the middle. This is necessary due to the construction of the linear shaker.

c) The tube is loaded into the shaker and allowed to agitate for 45 minutes.

d) The tube is unloaded from the shaker, uncapped, 20 mls of extraction solvent is added, the tube is capped, weighed, the grip is changed and finally loaded into the shaker to be agitated for 45 minutes.

e) The tube is removed from the shaker and loaded into a holder in front of the centrifuge.

f) A balance tube is removed from the centrifuge and loaded into a second holder in front of the centrifuge.

g) The sample tube is removed from the first holder and loaded into the centrifuge where a 3 minute spin is now initiated.

h) While spinning, the robotic arm will perform a number of tasks:

i) remove a gravity slip cap from a holding tube
ii) remove a holding tube from it's rack.
iii) weight the empty holding tube
iv) load the holding tube into the prep-sep workstation
v) change heads
vi) remove a prep-sep cartridge from the prep-sep dispenser
vii) load the prep-sep cartridge into the prep-sep workstation
viii) wash the prep-sep cartridge
ix) change heads
x) wait for the spin to complete

7. When the spin is complete, the arm will:

a) unload the sample tube from the centrifuge into the first holder
b) re-load the balance tube from the second position into the centrifuge
c) remove the sample tube from the first holder
d) uncap the sample tube
e) place the sample tube in the balance and store it's weight uncapped
f) leave the tube in the balance and pickup a cannula hand
g) position the cannula over the sample tube
h) find the surface of the solvent phase
i) calculate an offset from the surface such that at least 90% of the extraction solvent is removed
j) draw the solvent back into a 25 ml syringe on the MLS
k) position the cannula over the prep-sep in the prep-sep station
l) dispense the solvent into the prep-sep cartridge
m) force the solvent thru the prep-sep packing into the holding tube.
n) remove the holding tube from the prep-sep station and return it to it's rack

o) wash the cannula and syringe. Dispose of the used prep-sep
p) cap the holding tube with a gravity slip cap
q) prefill a syringe with a second extraction solvent portion
r) remove the tube from the balance and dispense the second solvent portion into the tube
s) cap the tube, shift the grip and load it into the shaker, where it will agitate again for 45 minutes

8. Upon completion of the second agitation, steps 3, 4, 5, 7 (a)-(j) are repeated.

9. The robot will then continue operations as follows:
   a) dispose of the used prep-sep cartridge
   b) remove the holding tube from the prep-sep workstation and load it into the vortexer
   c) vortex the combined extracts for 2 minutes

10. While vortexing, the following steps will be performed:
   a) the robotic arm will unload the sample tube from the balance
   b) the contents are aspirated to a waste reservoir
   c) the sample tube is capped and returned to it's rack

11. When vortexing is complete, the following sequence will occur:
   a) unload the holding tube from the vortexer and load it into the balance
   b) record the weight of the tube and the combined extracts
   c) pickup and position the cannula tip to 0.5 cm above the surface of the combined extracts.
   d) find the liquid surface
   e) calculate an offset
   f) draw a 1 g aliquot of solvent into the 25 ml syringe
   g) dispense this 1 g aliquot to an output tube
   h) wash the cannula and syringe
   i) remove the holding tube from the balance and replace it in the rack
   j) cap the holding tube with a gravity slip cap

This outline lists the robotic steps performed on a sample in a sequential manner. However, to use each work station to it's greatest potential (i.e. minimize idle time) the system will be required to run in a serialized mode. This procedure requires that modules such as the shaker and centrifuge operate independantly of the arm, so in effect the system idle time is minimized. This was accomplished by fine-tuning the timing of each step so that synchronization between the arm and the modules was achieved.

In addition, at critical steps such as capping and getting a pippette tip,

confirm operations are carried out to ensure the proceeding step was successful before the subsequent operation is carried out.

SERIALIZATION OF THE ROBOTIC PROCEDURE

From the documention supplied by Zymark, an algorithm was devised to serialize this application. The basic method is defined as follows:

Robotic Step: an activity where the arm is utilized, i.e.: capping tubes, moving tubes around the table.

Non-Robotic Step: an activity where the arm is not utilized, i.e.: a tube being agitated, spun in the centrifuge.

In this development work, the robotic control program is divided into sections, terminated by a non-robotic step and the times to complete these sections are obtained. For example:

Section I	-	Get sample tube	0.8 min
	-	Load into shaker	
Section II	-	Add first portion of extraction solvent	4 min
	-	Load shaker	
Section III	-	Unload shaker	17 min
	-	Centrifuge sample	
	-	Pipette off solvent phase	
	-	Add second extraction solvent portion	
	-	Load shaker	
Section IV	-	Unload shaker	21 min
	-	Centrifuge sample	
	-	Pipette of second solvent phase	
	-	Aspirate remaining contents to waste receptacle	
	-	take 1 g aliquot	
			42.8 min

Therefore Total Robotic Time/Sample = 42.8 min = .7 hours

Therefore Sample Output Rate = $\frac{1 \text{ sample}}{.7 \text{ hours}} \times 24 \text{ hours} = 33 \text{ sample/day}$

To minimize programming complexity, centrifuge, although by definition a non-robotic step is turned into a robotic step by utilizing the arm while the sample is spinning, i.e.: by setting up the prep-sep station.

Since the robotic time per sample is fairly close to the 1 hour sample agitation time, the procedure is time-balanced against robotic time, rather than agitation time. If the procedure were balanced against a strict requirement for a 1 hour agitation time, there would be a dead time of 17.2 minute/sample, which implies a drastic reduction in throughput.

However, this change in time could only be justified if it had no adverse effect on recoveries, which was tested by running spiked and real samples through the system.

SOFTWARE

A major limitation of the Zymate II system is it's 60K memory for the system dictionary, programs and data. A memory upgrade is not cost-effective since contract specifications called for a PC driven system anyway. To accomplish this, an IBM compatible P.C. was linked to the controller to provide:

i. a menu-driven user interface for the system.
ii. expanded data storage and processing capabilities to the system.
iii. enhanced error-recovery.
iv. uploading of data to other systems.

This way the controller functions as a transparent low-level module, while the P.C. functions as a high-level controller, responsible for data

reductions, file manipulation and repeat production.

During operation, the operator is presented with a main menu from which he or she can access to all phases of the robotic procedures. The system is broken into two phases:

1) Pre-digestion Routines

2) Extraction Routines

In the pre-digestion routine, the operator is presented with a form where he enters date, name, method, LIS # and workstation. He is then requested to enter the number of samples he wishes to digest. Sample attributes are entered next, in such a way that the number of keystrokes is minimized, i.e. with only sample codes. Upon completion, the empty tubes are first weighed and stored in a data file. The operator will then load the samples into the tubes. The rest of the procedures will be handled by the robotic system.

In the extraction routine, data entries and manual intervention are minimal. In the beginning of the run, the operator will review a checklist shown on the terminal to ensure the robot has sufficient supply of disposable sep-pak cartridges, solvents and reagents for the duration of the run. The rest of the operation again will be automatic.

EVALUATION OF THE ROBOTIC SYSTEM AND PROGRAMMING CONSIDERATIONS

During the course of the method development, a number of problems were encountered when trying to convert the manual method to the robotic method. Some examples are presented below:

(i) one of the contract specifications required was that all materials to be in contact with the extracts were to be made of stainless or Teflon. However, the Teflon coated caps for the 40 ml centrifuge

tubes were found to leak excessively when loaded on their sides in the linear shaker. Increasing capping torque had the effect of overtightening the caps such that they could not be uncapped by the arm. To solve this problem, extensive modification of the existing capping software was carried out to eliminate capping error. In these procedures, the robotic hand would test the torque of the cap. If the required torque could not be achieved, the robot would try two more times to tighten the cap and check for leaking by the weight difference.

(ii) Corrosion in the system was severe among metallic parts in contact with acid. In this work, the metal feed tubes in the dispensing post were removed and replaced with a Teflon tubing to eliminate this problem. However, cannular tip was also found to be corroded and consequently led to a loss of pipetting efficiency. Valves in contact with acid and solvent had to be frequently replaced during our development process.

(iii) The Zymark centrifuge consisted of rubber bungs in the bottom of the bucket. After centrifugation of samples, vaporized solvent would stick onto the rubber, forming good seal with the bottom of the centrifuge tubes. After a spin, the bucket would stay attached and be pulled out of the centrifuge which would lead to a collison. The solution was to insert a Teflon coated septum of proper diameter into the bucket , which eliminate the bonding of the rubber with the tubes.

(iv) Because there is an element of potential mis-alignment in the system, (e.g. racks, the shaker, centrifuge buckets, balance tube holders etc. where tubes can move slightly,), it was necessary to develop and incorporate checking steps into all routines which will affect

throughput slightly, but result in less failures.

(v) Initial design included the use of 10 ml disposable plastic pipette tips to withdraw the upper layer of solvent from a centrifuge tube. However, due to variations in tube volume and sample size, the surface level of the extraction solvent will vary. Thus, the routine to find the initial starting point at which to begin a pipette operation is necessary. It was also discovered that the pipette tips are not perfectly straight and so may touch the wall of the tube. This results in an increasing weight reading during pipetting. A cannula tip was then subsequently used, which solved the pipetting problem and gave precise weights. However, this required a wash station of it's own and addition steps for transferring.

(vi) Depending on the type of fish, emulsions did form in some samples at high shaker speeds, which could not be broken by the centrifuge in the time alloted. Adjustments were made to shaker speed, centrifuge speed, to minimize this problem.

(vii) Pipetting more than 95% of solvent from top of acid phase was found to be very difficult. For this reason, the samples was extracted at least two times, which minimize the loss of solvent. In addition, several checking steps were incorporated into the pipetting software to compensate this problem.

(viii) Variations in glassware size required extensive sample abort programming, due to the probability of failure in an operation. Theoretically, every move should be confirmed on systems such as this. However, this would also increase the complexity of the software, decrease the maintainability of the completed code and fall outside the time constraints of the project. Compromises were therefore made

in the design of the software; operations with a high probability of failure, i.e.: capping were extensively confirmed; operations with a low probability on the other hand, were not as in the case of vortexer operations.

(ix) Variations in the sample matrix (fish tissue) e.g. water content and lipid content, required some development of an appropriate pipetting algorithm. Attempts were made to define cannula pipette tip position over the acid/solvent interface as a function of sample weight. The interface level, however, varied too much for this approach to be of any practical use. By assuming negligible miscibility of acid and solvent conbined with a top-down approach (quite literally in this case!), an algorithm was developed with the accuracy and reproducibility surpassing that of a manual method. An optical sensor approach to measure interface height was also considered, but abandoned upon advice from Zymark, who had previous experience with these systems.

(x) Initial runs had low recoveries due to a discreprency in the robotic specifications and the manual method. All manually extracted samples were allowed to digest overnight, whereas the robotic method explicitly stated 1 hour digestion time. This necessitated a major redesign and rewrite of the completed code

The final phase of this project involved a full and comprehensive analytical evaluation of the completed robot extraction workstation. This evaluation included extraction of 50 samples of each of:

1) Blanks (reagent only, no biological tissue present)
2) Spikes (fortified samples as per MOE technique)
3) Duplicates (multiple extractions of same tissue homogenate).

All the extractions have so far been completed, however, at the point of writing, the complete data are not fully avaliable. Since improved precision was one of our expectations of robotic automation, we had initially carried out a number of experiments to evaluate the reproducibility of the system. The results from these experiments are presented in Table I. Table II also tabulates the results of a comparison study of spiked fish samples between the manual and robotic method. The manual extraction and the final gas chromatographic analysis of the extracts were conducted in the MOE laboratory to give an accurate comparison of the developed robotic method and the manual method currently used in MOE laboratories. Review of the data indicates that the robotic method is as good or better than the manual method. In terms of reproducibility, the results are quite satisfactory.

CONCLUSION

In summary, the robotic workstation developed for the extraction of the fish tissues has demonstrated sufficient accuracy, precision and reliability as compared to the manual method. Although it is a complicated laboratory robotic application, statistical evaluation reveals that the method is quite reproducible and the overall sample throughput surpassing the requirement set by the Ministry of the Environment (33 samples/day).

Table I

COMPOUND	ACTUAL STD. CONC.	DETECTED BLANK SPIKE CONC.	MEAN DETECTED CONC. *	OVERALL % REC. **	STANDARD DEVIATION	C.V. %
PCB	1000	997.50	989.40	98.90	82.90	8.40
HCB	10	10.50	13.80	137.00	2.60	19.10
HEPTACHLOR	20	21.50	22.70	113.50	3.10	13.80
ALDRIN	40	49.50	45.10	112.80	5.00	11.00
PP-DDE	90	108.00	94.20	104.70	16.00	17.00
MIREX	100	138.50	146.40	146.40	33.50	22.80
ALPHA-BHC	40	25.80	38.80	97.00	7.60	19.80
BETA-BHC	40	33.80	46.70	116.80	8.20	17.60
GAMMA-BHC	40	34.50	48.70	121.80	8.70	17.80
ALPHA-CHLORDANE	40	36.80	40.20	100.50	3.93	9.80
GAMMA-CHLORDANE	40	32.30	34.80	87.00	3.22	9.20
OP-DDT	40	27.50	27.80	69.50	6.20	22.30
PP-DDD	40	33.50	37.70	94.30	4.55	12.10
PP-DDT	40	35.00	32.70	81.80	6.40	19.50
OCTACHLOROSTYRENE	20	21.30	22.30	111.50	4.07	18.30
P1BHCD	40	34.00	35.20	88.00	6.098	17.30
P1CHLN	40	35.80	34.30	85.50	7.62	22.20

Table II

COMPOUND	MEAN		STANDARD DEVIATION		C.V.(%)	
	MANUAL	ROBOTIC	MANUAL	ROBOTIC	MANUAL	ROBOTIC
PCB	911.40	989.40	148.80	82.90	16.30	8.40
HCB	16.90	13.80	7.30	2.60	43.30	19.10
HEPTACHLOR	19.90	22.70	4.90	3.10	24.50	13.80
ALDRIN	47.70	45.10	7.70	5.00	16.20	11.00
PP-DDE	133.60	94.20	21.20	16.00	15.90	17.00
MIREX	130.90	146.40	17.40	33.50	13.30	22.80
ALPHA-BHC	20.00	38.80	6.20	7.60	31.10	19.80
BETA-BHC	37.70	46.70	10.00	8.20	26.50	17.60
GAMMA-BHC	31.00	48.70	8.80	8.70	28.40	17.80
ALPHA-CHLORDANE	27.20	40.20	6.80	3.93	24.90	9.80
GAMMA-CHLORDANE	26.20	34.80	6.70	3.22	25.70	9.20
OP-DDT	18.90	27.80	10.40	6.20	54.70	22.30
PP-DDD	25.50	37.70	6.30	4.55	24.90	12.10
PP-DDT	23.20	32.70	5.60	6.40	23.90	19.50
OCTACHLOROSTYRENE	28.30	22.30	7.80	4.07	27.50	18.30
P1BHCD	N/A	35.20	N/A	6.10	N/A	17.30
P1CHLN	N/A	34.30	N/A	7.62	N/A	22.20

CHARACTERIZATION OF THE FECAL INDICATOR BACTERIAL FLORA OF SANITARY SEWAGE WITH APPLICATION TO IDENTIFYING THE PRESENCE OF SANITARY WASTE IN STORM SEWERS.

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ABSTRACT

Samples were collected from surface runoff as well as from specific sites in sanitary sewer lines and in priority and nonpriority storm sewers during periods of wet and dry weather. Densities of fecal coliforms, Escherichia coli, fecal streptococci, enterococci, Pseudomonas aeruginosa, Clostridium perfringens, and Bifidobacterium were determined in each of the samples. Over 2,000 fecal streptococcal and 1,400 P. aeruginosa isolates were characterized further by biochemical testing, serotyping, or genotyping. Streptococcus faecalis var. liquefaciens and S. faecium were shown to be uniformly distributed among the high priority and nonpriority storm sewage and sanitary sewage samples. Streptococcus faecalis var. faecalis, on the other hand, was more prominent in the sanitary and high priority sewage. It is noteworthy that a high proportion of these S. faecalis isolates produced an acid curd in litmus milk. It would appear from the data collected that although P. aeruginosa densities were markedly higher in sanitary and high priority storm sewage, serotyping of the isolates does not help to identify sources of pollution. For example, 06 was the predominant P. aeruginosa serotype isolated from all samples. Genotyping results may yield more information. As might be expected, changes in bacterial indicator populations were best observed during periods of dry weather. Under dry conditions all indicator organisms, including Bifidobacterium sp., were present in higher levels in samples taken from the high priority storm sewage in comparison with the nonpriority samples. These preliminary results suggest that the characterization of specific indicator bacteria can be used to facilitate the tracing of illegal sanitary connections to priority storm sewers in the Metropolitan Toronto area.

Introduction

Storm sewers are designed to channel storm water from urban areas into surface waters. The content of storm sewers should be similar to direct runoff and the flow restricted primarily to storm events. A small amount of dry weather flow is expected in storm sewer lines due to ground water intrusion and activities such as lawn watering. However, a significant dry weather flow, coupled with high fecal coliform counts, strongly suggest that there are illegal sanitary connections somewhere in the storm sewer line. Since storm sewage is not normally treated prior to entering receiving waters, such as the Lake Ontario beach areas, any illegal connections would pose a potential health hazard.

The Ministry of the Environment has designated storm sewer outfalls discharging more than 1 L/sec. during dry weather periods and exhibiting fecal coliform (FC) densities of greater than 10,000 FC/100 mL as high priority and has called upon municipal agencies to identify and eliminate the source of the fecal pollution in these sewage lines. Although there has been some success, compliance with the Ministry's directive can be difficult due to the fact that good methods for source determination are lacking. For example, Escherichia coli, which is an excellent indicator of fecal contamination, does not lend itself to source determination because of its wide-spread occurrence in all fecal material. The fecal coliform to fecal streptococcus ratio also has some shortcomings in that the enumeration methods currently in use in Ontario are different from those originally employed by Geldreich and Kenner (1969). In addition, it has been shown that certain animal hosts, such as dogs, gulls and pigeons, exhibit ratios similar to humans (Seyfried, Harris, and Young, 1986 unpublished data), and that the ratios obtained from polluted waters change over time as a result of environmental stress (Seyfried, Harris and Young, 1986 unpublished data;

Feachem, 1975).

Recently, newer methods of source determination have been proposed. A study conducted at the University of Toronto (Seyfried, Harris and Young, 1986 unpublished) found that differences in the relative proportions and biotypes of group D streptococci existed between human and animal hosts. These findings are also supported by previous workers (Weather, et al 1979; Kenner 1973; and Geldreich 1976). Hill et al (1971) found that human subjects fed on a mainly mixed western diet carried a higher percentage of S. faecalis biotypes. Mundt (1982) also found S. faecalis to be the predominant species of group D streptococci in humans and was able to demonstrate marked differences in the litmus milk reactions of S. faecalis from humans, animals and plant material (Mundt, 1973).

Characterization of Pseudomonas aeruginosa isolated from waste-waters may also be of significant value in source determination because this species is found primarily in human as opposed to other domestic animal wastes, i.e. dogs and cats (Seyfried, Harris and Young, 1986 unpublisned data; Wheiter et al 1979), and because serotyping allows for sub-speciation of this organism into approximately 17 different heat-stable somatic antigenic groups (Kusama, 1978). Source tracing of Pseudomonas aeruginosa infections by serotyping has been used successfully in many clinical trials (Young and Moody, 1974; Baltimore et al. 1974). The method has also been used to trace the source of infection in swimmers (Seyfried and Fraser, 1978) and could possibly be adapted to tracing studies in other environmental settings.

It has been suggested that Bifidobacterium spp. are good indicators of human fecal wastes in surface waters (Buchanan and Gibbons, 1974; Levin, 1977; and Resnick and Levin, 1981). Bifidobacteria are present in concentrations of 10^9 organisms per gram of feces in humans (Geldrich, 1979), but have a very limited

distribution among other animals (Mara and Oragui, 1983). They have also been recovered from raw sewage (Resnick and Levin, 1981). Mara and Oragui (1983) reported that sorbitol fermenting species of bifidobacteria were exclusive to human fecal wastes and proposed a membrane filtration method for recovering bifidobacteria from surface waters.

In order to determine the relative merits of the novel source determination methods and to devise a methodology for the detection of human fecal wastes in storm sewer lines, a study to characterize the bacterial populations found in urban storm and sanitary wastes was initiated in the fall of 1986. Fecal indicator bacteria were enumerated in sanitary, storm, and priority storm sewage, and storm water runoff. Fecal streptococci and Pseudomonas aeruginosa were isolated and characterized with respect to the phenotypes, serotypes, and genotypes present in the different samples. Bifidobacteria were recovered from storm and sanitary wastes and the isolates tested for sorbitol fermentation.

Methods

Sampling Sites

The Mount Steven Trunk storm sewer, that discharges into the Don River, was selected as a representative of priority storm sewage. For comparative purposes, samples were collected from Mount Steven Trunk storm sewer branch lines that had been designated as non-priority storm sewers. A nearby sanitary sewer was also sampled in the same geographic area as the priority storm sewer sampling points. The location of all sampling points are described in Table 1 and shown schematically in Fig. 1.

Sample Collection and Analysis

Triplicate samples from outfall D-34 Mount Steven Trunk storm sewer were taken at three in-line points starting from an area close to the source of the suspected contamination and continuing downstream to a sampling point near the outfall. Triplicate samples were also taken from a nearby sanitary sewer at three in-line points adjacent to the priority storm sewer sampling points. Both sewers were sampled at these sites during the two dry weather surveys. The first of these surveys occurred in October-November, 1986. Triplicate samples from the three sampling points in both sewers were collected over 4 days and analyzed for fecal coliforms (M-TEC Agar) (Dufour, 1981), Escherichia coli (M-TEC urease treatment) (Dufour, 1975 and 1981), fecal streptococci (m-Enterococcus Agar) (Slanetz and Bartley, 1977), Enterococci (m-ME Agar) (Dufour, 1980) and Pseudomonas aeruginosa (mPA Agar) (Standard Methods, 1985). Selected samples were also enumerated for Bifidobacterium spp. (YN-17) (Mara and Oragui, 1983).

The second dry weather survey was conducted in June 1987. In addition to the above described sampling points, samples were also taken from two branch lines connecting with the main sewer trunk. Neither of these branch lines were suspected of having illegal sanitary connections because they had previously exhibited low fecal indicator levels. Triplicate samples were again collected over 4 dry weather days and analyzed as previously described. Clostridium perfringens (CPM-2) (M.O.E. 1986) was added to the aforementioned parameters and was isolated using a medium originally developed by Cabelli (1979), and modified by the Ministry of the Environment Southeastern Region Laboratory.

A wet weather survey of non-priority storm sewage and storm water runoff was conducted on July 14, 1987. Storm sewage was taken from two branch lines connecting

to the Mount Steven Trunk sewer. Previous sampling of one of these two branch lines had been conducted during dry weather survey 2. Storm water runoff was collected from street gutters on July 13 and 14. The site descriptions for the wet weather sampling points are listed in Table 1 and Figure 1. Wet weather samples were analyzed for the same parameters as during the second dry weather survey including analyses for Bifidobacterium and, where sample volume permitted, Clostridium perfringens.

A total of 2025 fecal streptococcal isolates were recovered on m-Enterococcus agar and m-ME agar from the two dry weather and one wet weather surveys. These were biochemically identified according to a test scheme developed by the Ministry of the Environment (Seyfried, Harris and Young, 1986 unpublished). Isolates identified as S. faecalis varieties were further tested for their reaction in litmus milk broth (Difco) according to the method of Mundt (1973).

A total of 1,402 presumptive target isolates of P. aeruginosa recovered on mPA were confirmed biochemically by Gram and oxidase reactions and characteristic reactions on skim milk agar (Brown and Foster, 1970). Further confirmation of P. aeruginosa was made utilizing acetamide agar slants (Standard Methods, 1985). Serotyping of the confirmed Pseudomonas aeruginosa isolates was performed using a Pseudomonas Antisera Kit (Difco) which allows for sub-speciation of the organisms into the 17 different heat-stable somatic antigen groups described by Kusama (1978). Bifidobacteria isolates obtained from dry and wet surveys were identified morphologically and biochemically according to the method of Buchanan and Gibbons (1974), which includes testing for sorbitol fermentation. Isolates of Bifidobacteria were also obtained from the fecal samples of humans, dogs and cats during the fall of 1986 and summer of 1987. The isolates were obtained by membrane filtration of diluted fecal samples onto YN-17 agar.

Chromosomal restriction endonuclease analyses (genotyping) of selected streptococcal and Pseudomonas aeruginosa isolates obtained from storm and sanitary sewage and storm water runoff was performed utilizing the methodology of Bradbury (1984, 1985).

Results and Discussion

The concentrations of fecal indicator bacteria and Pseudomonas aeruginosa found in sanitary sewage, high priority and non-priority storm sewage, and storm water runoff are presented in Table 2. The results show that the concentrations of fecal indicator bacteria in the Mount Steven Storm Trunk were high during both dry weather surveys. Although levels of greater than 10,000 fecal coliforms per 100 mL occurred throughout the main line (sampling points A, B, and C), high densities were noted most frequently at sites A and B. The source of the suspected fecal contamination is thought to be near site A, but the high fecal coliform and E. coli levels exhibited in sample B suggest that a second contaminant input may occur somewhere in-line between points A and B. The concentrations of fecal coliforms, and more specifically of E. coli, in sample C (near the outfall) tended to fluctuate but were usually lower than at sites A and B. Concentrations of fecal indicator bacteria in sanitary sewage were consistently high, as would be expected in this type of sample.

The fecal coliform to fecal streptococcus ratios observed in the sanitary sewage samples in dry weather were all above 4, indicating human fecal input. The FC/FS ratios varied from day to day in the storm sewer line, but there was a tendency toward ratios of >4.0 at points A and B. At site C, the FC/FS ratios were always below 4 which would suggest that no source of contamination is present and that the area is being impacted upon by upstream pollution.

Pseudomonas aeruginosa was also present in high concentrations in the sanitary sewer. Although this organism was found to occur in only 12% of the human population (Sutter et al., 1967), it is consistently isolated from sewage and highly polluted surface waters (Bonde, 1963; Hoadley, 1967; Cabelli, Kennedy and Levin, 1976).

Pseudomonas aeruginosa was recovered from the high priority storm sewer (A, B, and C) during both dry weather surveys. The levels exhibited during the second dry weather survey were higher than those recovered in survey 1 (Table 2) possibly due to the fact that the second survey was conducted during the summer period as opposed to the late fall sampling of survey 1. Pseudomonas has been shown to exhibit regrowth in nutrient enriched waters at high temperatures (Hoadley, 1977).

Previous investigators have shown P. aeruginosa to be more indicative of human rather than animal fecal wastes (Wheater et al., 1978, 1979). The concentrations present at points A and B, particularly during dry weather survey 2, would suggest that the contaminate input at these sites is human in origin. Levels of Pseudomonas at point C tended to be lower than at points A and B, again confirming that the source of the contamination lies upstream of this point.

Pseudomonas aeruginosa was not isolated from the high priority storm sewer during the first dry weather survey on October 28. Rainfall on the previous evening may have caused a dilution effect in the sewer such that P. aeruginosa could not be detected. Any incoming fecal material from street runoff would be of non-human origin (eg. dog) (Geldreich, 1979). This would cause an increase in the fecal coliform, E. coli and fecal streptococcus concentrations in the sewer but would not necessarily increase P. aeruginosa concentrations since animal feces (i.e. dogs, cats, racoons) generally do not contain Pseudomonas (Seyfried,

Harris and Young, manuscript in preparation).

Bifidobacteria were recovered from both high priority storm and sanitary sewage during dry weather surveys 1 and 2. This organism is thought to occur primarily in human wastes (Mara and Oragui, 1983) and was found in concentrations of 10^8 to 10^{10} in the human feces analyzed as a separate facet in this study. The high concentrations of this bacterium in the storm sewer at points A and B and the lower concentrations found at point C again confirms that human fecal input occurs at the two upstream points because this organism exhibits rapid die-off in surface waters (Oragui, 1982).

Clostridium perfringens was included as a parameter during the second dry weather survey. Current literature available on the bacterium suggests that it is associated with both human and animal wastes (Bisson and Cabelli, 1980; Geldreich, 1979). Generally the organism is regarded as a good indicator of fecal pollution in situations where there has been environmental stress due to disinfection, prolonged transit time or the presence of toxic wastes (Bisson and Cabelli, 1980; Geldreich, 1979; Fujioka and Shizumura, 1985). C. perfringens was recovered from both high priority storm and sanitary sewage, and was present in higher concentrations in sanitary sewage although it could not be isolated from the human fecal samples assayed. According to Geldreich (1979), C. perfringens is found in concentrations of 10^6 to 10^7 per gram of human feces but is only present in 13 to 35 percent of the population.

Additional samples X and Y analyzed during the second dry weather survey were collected from branch lines connecting to the main trunk line (see Table 1 and Figure 1). These lines were thought to be non-priority storm sewage according to the City of Toronto Public Works Department. Sample X exhibited low fecal indicator counts as would be expected in non-priority storm sewage. The sample

also contained very low Pseudomonas and Bifidobacteria levels which would indicate the absence of human fecal input at this site. Sample Y , however, exhibited high concentrations of fecal coliforms and E. coli, and on June 12 the FC counts were greater than 10,000/100 mL. Concentrations of Pseudomonas aeruginosa and Bifidobacteria were also high in this sample suggesting that a contaminant input, possibly of human origin is impacting on the sewer at or near this site. Although site Y is located upstream of site B, (see Figure 1) it cannot account for the magnitude of pollution exhibited at B since the bacterial concentrations recovered at Y were lower than those present at the B site. Because of its high fecal indicator bacterial levels, sample Y was grouped with the high priority storm sewage.

The FC/FS ratios exhibited in samples X and Y were always below 4. This would appear to contradict the evidence of human fecal input at site Y, i.e. Bifidobacteria concentrations. Previous studies however, have shown that FC/FS ratios tend to fluctuate over time as a result of the different die-off rates of the two bacterial groups (McNeil, 1985). It may be that the fecal pollution input impacting on the sewer at site Y is located in-line above this sampling site. Since Bifidobacteria also tend to die-off rapidly (Orajui, 1982), it would be interesting to determine if samples taken above site Y in this branch line exhibited an increase in this organism as well as an increase in the FC/FS ratio and levels of other fecal indicator bacteria.

Fecal indicator bacterial concentrations recovered from non-priority storm sewage and storm water runoff during wet weather (Table 2) revealed that street runoff is heavily contaminated with fecal material and contributes greatly to the contamination in storm sewer lines during storm events. The rainfall for this event commenced on the afternoon of July 13 and continued until the late

morning of July 14. Samples Q, X and Z were collected after the first flush had occurred. Sample P was collected at the start of the rainfall event and this may account for the higher Pseudomonas levels exhibited in this storm water runoff sample over that of storm water sample Q. As well, the fact that sample P was collected from a different geographical location (see Table 1) may account for this difference.

Allen Gardens is an area where gulls and pigeons feed and both of these bird species were found to carry Pseudomonas (Seyfried, Harris and Young, 1986, unpublished data). During wet weather, Pesudomonas aeruginosa concentrations in storm water and storm sewage may also be increased by runoff from vegetation (Hoadley, 1977).

The fecal coliform to fecal streptococci ratios exhibited in storm water and storm sewage during wet weather survey 1, were all below 4.0 suggesting fecal input of non-human origin. It would appear that FC/FS ratios may still be a useful diagnostic tool for source determination in storm sewer lines when applied close to the input, although many investigators cautioned its use in surface water analyses (Wheater et al., 1979; Palmer 1984; Diebel 1964).

Bifidobacteria were recovered from non-priority storm sewage samples X and Z during wet weather. Concentrations exhibited in sample X were much higher than those recovered during dry weather at this site. Our studies showed that animals such as dogs and cats can carry Bifidobacteria in their feces.

An example of the distribution of fecal streptococcal populations recovered from storm and sanitary sewage during dry weather survey 2 is given in Table 3. Streptococcus faecium comprised the greatest percentage of the fecal streptococcal group found in storm sewage and sanitary sewage during dry weather with the exception of samples B and Y (Dry Weather 2) and F (Dry Weather 1 and 2) which

exhibited higher recoveries of S. faecalis varieties. Seyfried, Harris and Young (1986 unpublished data) found S. faecium to be predominant in human fecal samples as well as several animal species (i.e. dogs, racoons). Studies by Wheater et al. (1979) have also shown S. faecium to predominate in human feces and sanitary sewage. However, other workers (Cooper and Ramadan, 1955; Kenner, 1978; Kjellander, 1960; and Mundt, 1982) have found a greater percentage of S. faecalis varieties. Dietary differences have been shown to account for variations in the streptoccal flora of the human intestinal tract (Hill et al., 1971; Finegold et al., 1975). There is a tendency for S. faecium to survive for longer periods than S. faecalis in polluted waters (Dufour 1985 personal communication) which may account for its greater recovery from storm and sanitary sewage. Within the S. faecalis group there was some variation in recovery of S. faecalis var. faecalis and S. faecalis var. liquefaciens in the storm and sanitary sewage samples, with both varieties predominating during different dry weather surveys and at different sample points in both sewers. Other workers have found S. faecalis var. faecalis to be dominant in human feces (Kenner, 1978). Geldreich (1979) found that S. faecalis var. liquefaciens was present in only 26 percent of the human population and could be recovered from environmental sources.

Sample X (non-priority) exhibited low percentages of S. faecalis varieties and showed a higher percentage of S. faecium var. casseliflavus an organism which is found on vegetation (Mundt and Graham, 1968) and in the feces of animals such as geese (Seyfried, Harris and Young, 1986, unpublished data). S. faecium var. casseliflavus was also recovered at high percentages in storm water and storm sewage during wet weather but was generally not recovered from sanitary sewage and has not been shown to occur in human feces (Seyfried,

Harris, and Young, 1986 unpublished data). Other streptococcal species not recovered in sanitary sewage but found in storm sewage were S. bovis and S. avium. Both of these species are found in animals such as dogs but not in human fecal material (Seyfried, Harris and Young, 1986 unpublished data).

The litmus milk reactions of S. faecalis varieties (Table 4) demonstrate that acid curd producing strains are common in sanitary sewage. Mundt (1973) found that over 90% of S. faecalis cultures from non-human sources such as animals, plants and insects gave proteinization reactions in litmus milk, while isolates from human feces produced an acid curd. Seyfried et al. (unpublished data) also found that isolates of S. faecalis from humans did not proteinize litmus milk. However, these same isolates also did not demonstrate the same ability to produce acid curd reactions.

Most of the isolates from sanitary sewage giving proteinization reactions were S. faecalis var. liquefaciens and were probably of non-human origin because this variety can occur on such environmental sources as plants (Mundt et al., 1959). Isolates from high priority storm sewage showed a high percentage of acid curd production, but this reaction was also given by some of the non-priority storm sewage isolates from sample X during dry and wet weather and by isolates obtained from storm water sample Q. More storm water and non-priority storm sewage S. faecalis isolates will have to be tested before the usefulness of this test can be assessed.

The greatest percentage of Pseudomonas aeruginosa serotypes in high priority and non-priority storm sewage, sanitary sewage and storm water runoff were serotype number 6 (data available upon request). Other serotypes common to all sample types were serotypes 1, 11, 4, 3, and 2. Previous research has shown most of these isolates to be common to both humans and animals (Habs 1957;

Sandvik 1960; and Verder and Evans 1961). Serotype 10 was found to be more prevalent in storm sewer sample Y than was serotype 6. This serotype was rarely isolated from non-priority storm sewage and storm water runoff and was isolated from sanitary sewage but not to the degree that would be expected if this serotype was common to human fecal material. Overall, the value of Pseudomonas serotyping as a means of pollution source differentiation appears to be limited and perhaps it would be more useful to look at Pseudomonas concentrations in storm sewage.

The percentage of sorbitol fermenting Bifidobacteria in human feces and sanitary sewage is high (Table 5). Mara and Oragui (1983) have found sorbitol fermenting species of Bifidobacteria, i.e. B. adolescentis and B. breve to be exclusive to human feces. Other workers (McNeil 1985) have also reported this fact. The presence of these organisms in human feces is also not effected by diet and geographical variation (Drasar 1974) as are group D streptococci.

Bifidobacteria isolates obtained from dog and cat feces did not ferment sorbitol. Although some of the non-priority storm sewage isolates gave sorbitol fermentation reactions not enough isolates were tested (i.e. sample Z) to make any definite conclusions about the results. Isolates from high priority storm sewage samples A and B showed somewhat higher percentages of sorbitol fermentation reactions. Sample Y, which was originally submitted as non-priority storm sewage showed a higher percentage of the sorbitol fermenting strains than high priority storm sewage samples A and B and as previously mentioned, this site could possibly be impacted on by a human sanitary input.

It would appear, based on these results, that sorbitol fermenting Bifidobacteria may be good indicators of human fecal contamination.

CONCLUSIONS

Although there is still more data to be collected for this study, some preliminary conclusions can be made at this time. These are:

1. Fecal contamination, most likely of human origin, is present in the high priority storm sewer line at or near sampling points A, B and Y.
2. Upstream contamination is impacting on the storm sewer at the downstream area C.
3. Street runoff during wet weather is highly contaminated with fecal material.
4. Bifidobacteria may be useful as indicators of human fecal wastes in storm sewage.
5. A high percentage of acid curd producing strains of S. faecalis may be indicative of sanitary wastes in storm sewer lines.
6. FC/FS ratios may be useful as a supplementary interpretive tool for source differentiation within storm sewer lines. This would have to be investigated further because the results were not consistant at all points.
7. High Pseudomonas aeruginosa concentrations in storm sewage may indicate the presence of human sanitary wastes but Pseudomonas serotyping is not applicable to source differentiation; and
8. Source determination during storm events cannot be accomplished with a high degree of accuracy.

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Table 1: Sampling Locations of High Priority and Non-Priority Storm Sewers, Sanitary Sewer and Storm Water Runoff.

Sample	Code	Description
High Priority Storm Sewer Line: Mount Steven Storm Sewer Trunk	A	Danforth and Jones Avenues - furthest in-line sampling point (near source of suspected pollution input.
	B	Pape and Strathcona Avenues (mid-line sampling point).
	C	First and Broadview Avenuse (near outfall).
Non-Priority Storm Sewer Branch Lines	Y	Chatham and Jones Avenues (connects to main line above sampling point B).
	X	Danforth and Woodycrest Avenues (connects to main line above sampling point A).
	Z	Pape and Cavell Avenues.
Storm Water Runoff	P	Parliment and Carlton Streets
	Q	Pape and Cavell Avenues
Sanitary Sewage Line	D	Danforth and Jones Avenues
	E	Strathcona and Pape Avenues
	F	First and Broadview Avenues

Table 2 Overall Geometric Mean Concentrations of Fecal Coliforms, E. coli Fecal Streptococci, Enterococci, Pseudomonas aeruginosa, Bifidobacteria and Clostridium perfringens Recovered from Sanitary Sewage as well as High Priority and Non Priority Storm Sewage During Dry and Wet Weather Surveys.

		Parameter Per 100 mL Sample						
Survey	Site	Fecal Coliforms	E. coli	Fecal Streptococci	Enterococci	Pseudomonas Aeruginosa	Bifidobacterium	Clostridium Perfringens
Dry Weather I[a]	Sanitary Sewage							
	D	2.0×10^6	7.1×10^3	2.0×10^3	2.0×10^3	1.2×10^1 *	-	-
	E	2.7×10^6	4.0×10^3	1.6×10^3	2.0×10^3	2.2×10^1 *	-	-
	F	3.9×10^6	2.2×10^3	2.2×10^3	8.5×10^2	4.6	-	-
	High Priority Storm Sewage							
	A	8.1×10^3	1.4×10^6	1.5×10^5	1.4×10^5	8.6×10^3	-	-
	B	1.3×10^4	1.5×10^6	1.9×10^5	9.4×10^4	4.5×10^3	-	-
	C	3.8×10^3	2.3×10^6	3.8×10^5	4.9×10^5	2.7×10^3 *	-	-
Dry Weather II[a]	Sanitary Sewage							
	D	2.0×10^6	1.9×10^6	1.5×10^4	5.0×10^4	1.0×10^4	3.7×10^4	2.3×10^4
	E	3.5×10^6	2.6×10^6	1.1×10^5	1.1×10^5	1.8×10^4	6.3×10^4	5.2×10^4
	F	1.7×10^6	1.5×10^6	2.75×10^5 *	2.4×10^5 *	8.4×10^3	7.1×10^4	1.0×10^4
	High Priority Storm Sewage							
	A	4.9×10^4	3.1×10^4	1.0×10^4	3.9×10^3	1.6×10^2	5.2×10^4	6.4×10^2
	B	8.5×10^4	6.2×10^4	8.0×10^3	2.4×10^3	6.3×10^2	1.4×10^5	4.4×10^2
	C	9.3×10^3	6.0×10^3	4.8×10^4	1.3×10^4	9.3×10^1	1.5×10^3	7.5×10^2
	Y	3.8×10^3	2.9×10^3	4.0×10^3	2.05×10^3	3.9×10^1 *	5.2×10^2	5.35×10^2
	Non Priority Storm Sewage							
	X	1.3×10^3	9.0×10^2	3.5×10^3	2.1×10^3	6.75	1.2×10^1 *	1.2×10^2
Wet Weather I[b]	Runoff							
	P	5.8×10^3	4.8×10^3	2.4×10^4	-	5.6×10^2	-	-
	Q	6.3×10^3	2.9×10^3	3.0×10^3	-	4.6×10^1	-	-
	Non Priority Storm Sewage							
	X	1.3×10^5	8.6×10^4	8.9×10^4	9.8×10^4	1.5×10^3	2.4×10^3	1.3×10^3
	Z	4.1×10^4	1.1×10^4	1.2×10^5	7.1×10^4	5.1×10^2	4.4×10^2	-

* Approximate Data [a] sampled over a 4-day period; [b] sampled over a 1-day period

Table 3. Fecal Streptococcus Populations Recovered From Storm and Sanitary Sewage During Dry Weather Survey 2, 1987.

Species and variant	High Priority Storm Sewage				Non Priority Storm Sewage		Sanitary Sewage	
	A	B	C	Y	X	D	E	F
S.faecalis var.								
Faecalis	8(6.95)	41(34.7)	15(12.8)	12(25.5)	3(5.3)	19(13.4)	9(10.5)	15(26.9)
Liquefaciens	4(3.5)	12(10.2)	16(13.7)	10(21.3)	4(7.1)	23(16.2)	16(18.6)	28(50.0)
Zymogenes	-	-	1(0.85)	1(2.1)	-	-	-	2(3.6)
S. Faecium	36(31.3)	32(27.1)	31(26.5)	5(10.6)	20(35.7)	87(61.3)	57(66.3)	10(17.8)
S.Faecium var. casseliflavus	31(26.9)	12(10.2)	15(12.8)	9(19.1)	26(46.4)	2(1.4)	1(1.2)	
S. durans	2(1.7)	2(1.7)	7(6.0)	3(6.4)	2(3.6)	10(7.0)	1(1.2)	
S. bovis	-	-	-	-	-	-	-	
S.bovis (A typ)	8(6.95)	3(2.5)	-	2(4.2)	-	-	-	
S. avium	13(11.3)	11(9.3)	8(6.8)	2(4.2)	-	1(0.7)	-	
F. S.	5(4.3)	-	9(7.7)	-	-	-	2(2.3)	
Non F.S.	7(6.1)	3(2.5)	8(6.8)	1(2.1)	1(1.8)	-	-	
S. lactis	1(0.9)	2(1.7)	7(6.0)	2(4.2)	-	-	-	1(1.8)

( ) Percentage

Table 4. Percentage of *S. faecalis* Isolates Producing Acid Curd or Proteinization Reactions in Litmus Milk from Dry Weather Survey 2, and Wet Weather Survey 1 Samples.

Survey	Sample	Total Isolates	Acid Curd	Alkaline Curd	Proteinization	No. Reaction
	High Priority Storm Sewage					
Dry	A	12	9(75)	-	2(16.7)	1(8.3)
Weather	B	53	41(77.4)	4(7.5)	4(7.5)	4(7.5)
Survey	C	32	17(53.1)	2(6.25)	11(34.4)	2(6.25)
2	Y	23	18(78.3)	-	4(17.4)	1(4.3)
	Non Priority Storm Sewage					
	X	7	2(28.6)	-	-	5(71.4)
	Sanitary Sewage					
	D	42	22(52.4)	-	7(16.7)	13(31)
	E	24	12(50.0)	-	12(50.0)	-
	F	45	26(57.8)	-	17(37.8)	3(6.7)
	Storm water Runoff					
	P	10	2(20)	2(20)	5(50)	1(10)
Wet	Q	18	10(55.5)	2(11.1)	1(5.5)	5(27.8)
Weather	Storm Sewage					
Survey	X	16	6(37.5)	1(6.25)	9(56.3)	-
1	Z	12	1(8.3)	5(41.7)	4(33.3)	2(16.7)

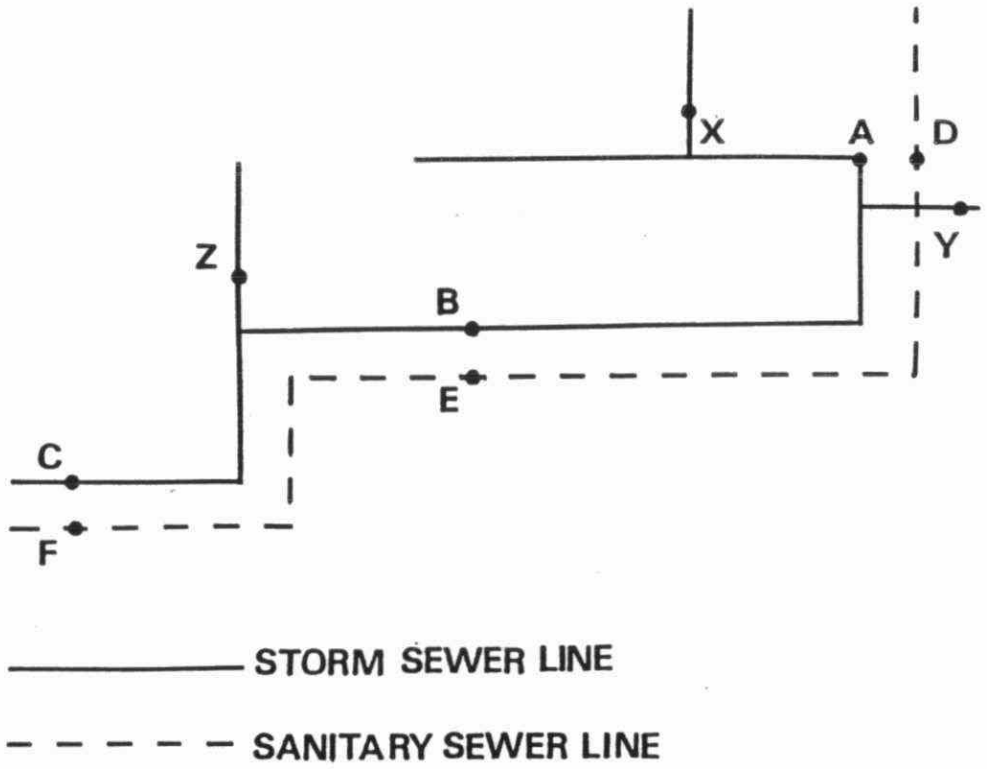
( ) Percentage

Table 5. Percentage of Sorbitol Fermenting Bifidobacteria in High Priority and Non Priority Storm Sewage, Sanitary Sewage and Feces

Sample	Source	Total Isolates Identified	Number of Sorbitol Fermentors
D,E,F	Sanitary Sewage	37	24(65)
A	High Priority	35	8(23)
B	Storm Sewage	29	4(14)
C		12	0
Y		32	13(41)
X	Non-Priority	15	3(20)
Z	Storm Sewage	5	2(40)
Feces	Human	38	23(61)
	Dog	24	0
	Cat	18	0

( ) Percentage

Figure 1: Schematic diagram of Mount Steven storm sewer and sanitary sewer lines



PATHOGENESIS OF NEOPLASTIC DISEASES AFFLICTING FERAL FISH

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ABSTRACT

Skin and liver neoplasms (tumors) affect bottom-dwelling fish in polluted areas of Lake Ontario and several other Great Lakes. Skin papillomas affecting white suckers (Catostomus commersoni) were examined for their behavior in the laboratory. After 12 weeks in the laboratory, 60% of the skin papillomas disappeared, but 41% of the surviving suckers developed new tumors. Neoplasms which disappeared may have been transient proliferative lesions which were dependent on the polluted environment for their continued existence. Papillomas which appeared in the laboratory suggest that pollution is not the only factor capable of initiating papilloma formation. Viruses were not found in similar papillomas with electron microscopy, but might be responsible for such papillomas. The liver tumors affecting many of these same fish are super-imposed upon a chronic inflammatory disease of the bile ducts. The histology and serum biochemistry of affected fish suggested that this condition might be due to chemical damage to bile ducts. The chemical alpha-naphthylisothiocyanate was used to induce bile duct necrosis in rainbow trout (Salmo gairdneri) to identify a serum biochemical indicator of bile duct disease in trout. Alkaline phosphatase (AP) levels in the serum correlated with this necrosis. Serum levels of AP in wild white suckers with variable levels of bile duct disease were poorly correlated with bile duct disease. This might reflect differences in chronic disease in the wild fish compared to acute bile duct necrosis and bile stasis in the rainbow trout. Serum biochemistry of white suckers with bile duct neoplasms were not significantly different from normal, indicating minor effects on liver function despite the presence of neoplasms. The absence of a suitable in-vivo marker for bile duct disease precluded studies of the persistence of both bile duct disease and tumors in the laboratory.

INTRODUCTION AND EPIDEMIOLOGY OF NEOPLASMS IN FERAL FISH

Cancers or tumors affect inordinately high proportions of many populations of wild fish inhabiting polluted areas of North America[1-10]. The frequency of tumors is often strongly correlated with sediment pollution[11], including polyaromatic hydrocarbons (PAH's) and chlorinated organics. Some chemicals have been identified as mammalian carcinogens[1,4,5-7], however their carcinogenicity at low-levels to fish has not been shown, nor have the effects of mixtures been investigated[12-14]. PAH's discharged by heavy industry have been implicated in the skin and liver neoplasms affecting brown bullheads (Ictalurus nebulosus) or white suckers (Catostomus commersoni) in the Black River in Ohio[1,2], Buffalo River in New York[4], the Niagara River[3], and Hamilton Harbour in Ontario [8,11]. It is often suggested that populations of wild fish can act as "sentinels" or "canaries" for the carcinogenic risks of adjacent human populations which are exposed indirectly to carcinogenic pollutants by eating the fish or drinking the water, or directly by occupational exposure in various industries[14,16,17].

Such suggestions assume that neoplasms develop in these fish in a manner similar to human cancer development, however very little is known about how neoplasms develop in fish. The biochemical and pathogenetic mechanisms important in the development of rodent neoplasms provide a basis for comparing the development of neoplasms in wild bullheads and suckers with neoplasms in laboratory species. We have examined the behavior of neoplasms affecting white suckers and serum levels of liver specific enzymes as a means of detecting fish with bile duct disease or liver tumors. The detoxification pathways important in the development of rodent

neoplasms[18,19] were also investigated in skin and liver neoplasms of wild fish.

SEQUENTIAL MODELS FOR CANCER DEVELOPMENT

Neoplasms induced with various chemicals and viruses in mammals have yielded several conceptual models for cancer development in the skin[19] and liver[20]. Histopathology[21,22] and enzyme histochemistry[23] of neoplasms of fish suggest similarities between carcinogenesis in mammals and fish. Our studies are evaluating the relevance of conceptual models developed to explain mammalian cancer development as a basis to understand the pathogenesis of skin and liver neoplasms in fish.

Chemically induced neoplasms originate with a mutation or change in the genetic material (DNA) of a cell which can be passed along to each successive generation of cells. The mutated cell is different from normal and this difference makes the new cell more or less competitive with its normal neighbours. If the mutation gives the cell a growth advantage it may multiply and form a small focus, or group of mutant cells, which are all different from normal neighbouring cells. Chronic exposure to chemicals that impair survival of normal cells selects for growth of mutated cells or foci which are resistant to toxic injury. Such resistant foci often contain increased levels of enzymes or new isozymes which protect it from the chemicals. This conceptual model for neoplasia development, the so-called "resistant" model[18,19], has been identified because investigators have available techniques to identify resistant cells and foci in mammalian models of carcinogenesis, and are able to determine the fate of such foci under a variety of subsequent conditions[24]. Decreased levels of mixed function oxidases and increased levels of various detoxification enzymes,

such as glutathione-S-transferase (GST) are important in resistance of foci to chronic toxicity[25,26].

Continued chemical toxicity forms the second stage of cancer development, promotion. Resistant cells continue to proliferate[27-29] eventually forming foci large enough to be called neoplasms. Promotion can also occur in response to chemicals such as PCB's that stimulate proliferation of altered cells, or in dietary conditions that affect the survival of normal cells. At this stage, if the promotional conditions (chemical toxicity) disappear, these cells can revert to normal and the neoplasm will regress. Continued promotion and cell division facilitates later steps termed progression, in which additional genetic changes remove these cells from host control over their growth. Uncontrolled growth may be due to oncogenes, which are mutated genes involved in the control of normal cell growth. It is presently uncertain if chemical damage results in these later changes in oncogenes. Progression leads to the formation of "malignant" cancers which continue to grow without promotion, invade adjacent areas of tissue, and "metastasize" to distant tissues, forming new colonies of cancers cells that eventually kill the host organism. All models for cancer development due to resistance require the initial mutational event, and differential survival or growth of mutated cells, although the mechanism for resistance may vary[31,32].

Resistance mechanisms have not been identified in altered foci, early neoplasms or cancers of fish, although they develop focal populations of phenotypically abnormal cells[23], which are typical of those seen in the rodent models. Progression of fish neoplasms to malignancies is rare. Fish neoplasms appear abnormal histologically and may invade adjacent tissue, but

they rarely metastasize to distant tissues or cause the death of the host. However, histological evidence of local invasion is evidence for progression towards malignancy in fish neoplasms and reflects the additive effects involved in initiation, promotion and progression of neoplasms. The mechanisms of initiation and promotion by chemicals in the early stages of neoplastic development in fish need to be understood in order to fully interpret the causes of naturally occurring neoplasms of suckers and bullheads. Accordingly, we are investigating the biochemical mechanisms for resistance in fish neoplasms, as well as characterizing the behavior and effects on the host of skin and liver neoplasms, which are the two most common neoplasms affecting wild fish in the Great Lakes.

SKIN NEOPLASMS AFFECTING WHITE SUCKERS AND BROWN BULLHEADS

Skin neoplasms affect up to 50% of the white suckers and brown bullheads in areas of Lake Ontario[15,33]. These epidermal papillomas closely resemble the benign epidermal papillomas which are induced in the sequential model for mouse skin cancers[19]. The fish papillomas rarely invade adjacent tissues, and are not reported to metastasize, suggesting initiation and promotion but no progression. Similar benign papillomas affect many fish species in unpolluted areas, often containing intra-cellular virus particles, which can act as both initiators and promoters of papilloma formation[34,35]. Epidermal papillomas also affect suckers and bullheads in unpolluted areas of the Great Lakes, and the failure to identify viruses with electron microscopy[35], does not preclude their being the etiological agent. Viruses are host specific and are unlikely to affect humans, suggesting that papillomas on fish in polluted areas cannot indicate a human risk. The reasons for higher frequencies of papillomas in populations from

polluted areas are unkown. Sediment extracts can induce similar epidermal papillomas on exposed bullheads[36], suggesting that chemicals may somehow increase the incidence of viral papillomas by a promoting effect.

Epidermal papillomas affecting white suckers from Oakville creek had normal or even reduced levels of GST[37], which is often a resistance mechanism in mammalian liver and skin neoplasms promoted by chemicals[18,20,24-26,30]. These papillomas likely have increased levels of some enzymes (glutathione reductase, glutathione peroxidase) that would protect them from the inflammatory cell response to an infectious agent[37]. The mechanism responsible for the high rates of papillomas is obviously not species specific because papillomas are common in both bullheads and suckers. If chemicals are responsible, they are unlikely to be a significant mammalian hazard because these fish have a mild degree of neoplastic progression, even though they are exposed to heavily polluted sediments. The absence of papillomas on trout and salmon in the same locations suggests that no risk is posed by the water above the sediments. The relative absence of invasive or metatstatic papillomas might suggest that mutagenicity is not affecting the papilloma incidence, because local invasion of papillomas is infrequent (4%) [15,34,35]. Promotion of skin neoplasms with mutagenic agents markedly enhances their progression to invasive malignancies[39].

Epidermal papillomas in white suckers from Oakville Creek were studied after removing the fish to our laboratory[40]. Papillomas (60%) which disappeared after 12 weeks were likely dependent on the locality (pollution?) for their persistence just as promoter-dependent stages of cancer development require additional mutations before becoming

independent[18,19]. The remainder of the papillomas persisted for the 12 weeks at the same size, suggesting they were independent of the polluted environment. New papillomas also developed on 41% of the survivors suggesting initiation and/or promotion in the absence of pollution. If persisting papillomas are caused by viruses, some are still apparently promoter-dependent, but most are not, as would be expected for viral induced papillomas. These findings, that papillomas of white suckers behave quite differently from chemically promoted skin papillomas of rodents, is consistent with our view that these skin papillomas in wild fish are not reliable evidence of exposure to carcinogenic pollutants.

LIVER NEOPLASIA: FIELD AND LABORATORY INVESTIGATIONS

Liver neoplasms affected 4% of the brown bullheads and white suckers from Hamilton harbour[15], 33-38% of the bullheads from the Black River in Ohio[1,2], and 6% of the white suckers from Oakville Creek[33,38]. Correlations between pollution and liver neoplasia in other locations[1-11], and evidence for the induction of liver neoplasms with carcinogenic chemicals[13,14,16], suggests that these neoplasms could be due to chemical carcinogens in the sediments. Fish from the Great Lakes generally develop neoplasms of the bile duct epithelium[1,2,15], while neoplasms induced in the laboratory and most other populations of wild fish develop from hepatocytes[13,14,16]. The bile duct epithelium of white suckers and brown bullheads taken from the Great Lakes is often hyperplastic and surrounded by inflammation which may pre-dispose the fish to bile duct neoplasms. A correlation exists between the severity of this condition and elevated serum bilirubin levels[41] (jaundice), consistent with morphological evidence of obstructive damage to bile ducts.

Invasive neoplasms of the bile ducts have been documented[1,2,15], indicating that initiation, promotion and progression leading to invasive malignancies is occurring. Initiation (mutation) of altered cells is facilitated by a high rate of proliferation[18,28,44]; the hyperplasia of bile ducts which characterizes this disease affecting these fish[15] likely is responsible for the propensity of bile duct epithelium to form early neoplasms. The bile ducts may be hyperplastic either because they are blocked, or more likely because they are excreting a high load of irritant chemicals metabolized by the liver. Chemicals such as those found in Great Lakes sediment[1,3,4,37] are oxidized and then conjugated by the liver and excreted as "detoxified" conjugates in the bile[45-47]. Bile stasis would expose the bile duct epithelium to these chemicals, which might undergo modifications[47,48] and cause mutations in the proliferating bile duct epithelium[48]. Continued hyperplasia would select (promote) cells which are resistant and possess a growth advantage when exposed to the bile contents, resulting in the benign neoplasms arising from bile duct epithelium[15]. Progression to carcinoma in these lesions suggests that additional genetic damage occurs more frequently in bile duct tumors than in the skin papillomas[1,2,15]. Total levels of GST are normal or reduced in benign and malignant bile duct tumors in white suckers[49], indicating GST is not important in the resistance of promoted bile duct epithelial cells.

The behavior of liver neoplasms after removal of fish from polluted locations is much more difficult to investigate than skin papillomas, which are easily detected in live fish. Attempts to identify a serum marker for proliferating bile ducts were inconclusive. Serum levels of alkaline phosphatase, which was released from bile duct epithelial cells during acute

necrosis[42], are not consistently correlated with the degree of bile duct disease identified at post mortem of affected white suckers[41]. Altered plasma proteins produced by neoplastic bile duct epithelial cells or hepatocytes are useful in detecting liver disease and neoplasms of rodents, and if similar in white suckers and brown bullheads, would permit in vivo diagnosis for behavioral studies[51-54]. Short-term carcinogenicity or analytical tests for carcinogenic activity provide a direct means of identifying the carcinogens excreted in bile of affected fish. Covalent binding assays have been developed to assess the role of various isozymes of GST and other detoxification enzymes in the protection of liver cells from various mutagenic chemicals. Primary cultures of rainbow trout hepatocytes[55], are being developed to investigate the types of activity present in bile from wild fish, and to compare the response of fish hepatocytes to those of rodents.

SIGNIFICANCE OF NEOPLASMS IN BOTTOM DWELLING FISH

Evidence for initiation, promotion and progression of liver tumors in some fish strongly indicated a chemical cause in sediments, and a possible risk for cancer development in exposed mammalian populations. The absence of neoplasms in mid-water species such as trout and salmon suggests that these chemicals are not transferred in drinking water or the fish food chain. We are comparing the susceptibility of different fish to carcinogens to see if the affected fish resemble mammals in their detoxification mechanisms, and are reliable indicators of human risk. Preliminary evidence suggests that wild fish are susceptible because they lack sufficient protective mechanisms which are present in mammals. These fish are unlikely to be a reliable indicator or sentinel of environmental pollution by human

carcinogens unless they have the same protective mechanisms.

CONCLUSIONS

Three and possibly more effects of chemicals are necessary for a cancer to develop, namely initiation, promotion and progression. Epidermal papillomas in white suckers from polluted areas of Lake Ontario fail to progress to invasive neoplasms suggesting that they are not caused by chronic exposure to mutagenic chemical effects on the skin. The persistence of some epidermal papillomas and emergence of new papillomas in the absence of environmental pollution and biochemical changes in papillomas are most consistent with a view that they are caused by infectious agents. Viruses are suspected but have not yet been found. The histology of bile duct neoplasms suggests that repeated exposure to mutagenic chemicals cause the progression of these neoplasms to invasive malignancies. Chronic bile duct disease and obstructive jaundice occurs in association with liver neoplasms in widely distributed populations of suckers and bullheads throughout the Great Lakes. Sediment-borne chemicals conjugated and excreted by the hepatocytes may be the major cause of these liver tumors, but definitive proof requires in vivo diagnosis of these diseases and evaluation of recovery after removal from polluted waters. Both epidermal papillomas and bile duct neoplasms contain normal or reduced total levels of GST, suggesting that the resistance mechanisms in these fish tumors are different from those in mammals. The role of various detoxificiation enzymes in protecting DNA and hepatocytes from mutagenic or toxic chemicals in sediments or bile need to be characterized in various affected and unaffected fish to see which are more representative of human susceptibility. The biochemical responses important in the development of

neoplasms provide a basis for comparing fish responses to mammalian responses, and will determine if fish are suitable sentinels for mammalian or human risks.

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Recombinant DNA Technology for Determining Source Inputs of Bacterial Pollution in Aquatic Habitats

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ABSTRACT

In a double-blind study, a total of 189 fecal Streptococci of human and non-human origin were classified by biochemical reactions, restriction endonuclease analysis (REA) and homology to streptococcal DNA probes derived from human and non-human sources. The host sources of the 189 isolates were human, gull, goose and unknown. Purified whole-cell DNA of each defined isolate was subjected to a Bam HI-REA and Southern blot analysis using the following fecal streptococcal-derived DNA probes: (1), two human-derived, total-chromosomal DNA probes 1410 and 1370, (2) one gull-derived, total-chromosomal DNA probe 1377; (3) two goose-derived probes - a 6.6 kilobasepair (Kb) Bam HI DNA fragment probe 807, and a total chromosomal DNA probe 1397. Results obtained with probe 1377 indicated that it was of human origin, though it was isolated from a gull host which had ingested it while feeding in human, fecal-waste polluted water. By contrast, results obtained with probes 1410, 1370 and 807, indicated that the probes were specific for their human and goose fecal streptococcal origins. Our results are presently being compared with those obtained by the MOE.

INTRODUCTION

Urban centres such as Toronto are confronted with significant pollution problems involving fecal bacterial contamination of their municipal water and associated drainage systems. This contamination is defined in terms of fecal coliform and fecal streptococcal counts (FC:FS ratio) which were originally used to distinguish between human fecal waste and animal (bird) fecal pollution (8,11). While this assay may be a useful indicator of the presence of water-borne pathogens, it is inadequate in determining the host source of the fecal waste, since to suggest that animal and human fecal material differ markedly in their FC:FS ratio, the differential survivability rates of coliforms and streptococci in aquatic habitats, as compared to the rates in the host gut, have all but been ignored. Furthermore, the close relationship between humans and animals in urban centres have resulted in a high degree of similarity between the intestinal flora of these hosts.

Studies involving biochemical characterization of fecal organisms have searched for host source-specific organisms. At present, however, biochemical assays lack the reliability and specificity to detect these organisms with the degree of predictability and accuracy required for tracing fecal pollution inputs to their sources.

Biochemical tests often detect temporary physiological conditions of the bacterial cell due to environmental forces that affect gene regulation resulting in unreliable classifications. By contrast, DNA analysis is more reliable because of the relative stability of the genome and therefore is a better method

for classifying fecal streptococci. In the present study we have utilized REA and Southern blot assays to identify the specific nature of each host as a source of fecal pollution.

The study was divided into two parts. Firstly, a Bam H1 REA was performed on 53 biochemically and source-defined fecal streptococci (generously provided by Dr. P.L. Seyfried, Department of Microbiology, University of Toronto).

Secondly, from this collection of defined strains, one gull isolate and one goose isolate were chosen as potential source-specific probes according to their REA patterns. In addition a 6.6 Kb probe (807) previously developed (6) as a goose-specific probe was also used. The DNA from two human isolates was used as whole chromosomal probes for use in the second half of the study.

The latter half of the project also involved a double blind study in collaboration with the MOE. A total of 189 fecal streptococci, that were biochemically and source defined by the MOE were sent to us for source identification as determined by REA and Southern blot assays. Although the results of the DNA study are presented herein, the final part of the study will involve further biochemical re-identifications of all 189 organisms prior to correlation of final results from both studies.

MATERIALS AND METHODS

Bacteria:

1. Fifty-three strains of fecal streptococci isolated from many different sources (humans, gulls, geese, pigs, ducks, muskrat,

and dogs) and biochemically defined as S. faecium, S. durans, S. bovis, S. faecium var. casselflavis, S. faecalis var. liquefaciens, S. faecalis var zymogenes, or S. faecalis var. faecalis were obtained in -70 C frozen cultures (Table 1). The latter were revived on BHI plates with overnight growth used to seed 4 ml BHI (Brain Heart Infusion, Difco Laboratories, Detroit, Michigan) broth cultures followed by 4 to 6 hr growth at 37 C.

2. The 807 isolate was an E. Coli strain of JM103 that had been transformed by a pUC-9 plasmid containing a 6.6 Kb DNA fragment inserted into the Bam H1 site of the vector.

3. The 189 streptococcal isolates were supplied by the MOE who knew both their source (human, gull, goose or unknown) and their biochemical definition. This information was not supplied to the present study. Similarly, our findings regarding the potential source of these organisms, as determined by REA and hybridization analysis, were not supplied to the MOE during their re-identification portion of the study. The 189 isolates were received on BHI agar under mineral oil.

4. The 1410 probe was derived from a human fecal streptococcal isolate which was a representative member of a group of 35 such isolates obtained from the fecal samples of non-laboratory workers as previously described (6). These isolates were biochemically undefined. In all cases, the bacteria were grown up from filter papers stored at -70 C.

5. The 1370 probe was derived from a human fecal S. faecium isolate which was a representative member of a group of six such isolates obtained from laboratory personnel. These isolates were biochemically defined.

Media:

The media used included BHI agar or broth used for culturing all fecal streptococci and LB plates containing ampicillin (Sigma Chemical Company, St. Louis, Mo.) at 40-50ug/ml. for growth of E. coli strain 807.

Chromosomal DNA Preparations:

All streptococcal chromosomal DNA was prepared according to the methods of Bradbury et al (2,3) with a few modifications. The incubation time of the BHI broth cultures at 37 C was reduced to 4 hours from overnight as it was easier to lyse cells with lysozyme than overnight cultures.

Plasmid DNA Preparations:

E. Coli plasmid DNA (strain 807) was prepared according to the method of Bradbury et al (1,4).

Restriction Endonuclease Digestion of DNA:

Bam H1 (Boehringer Mannheim, Ottawa, Ontario), which cuts streptococcal chromosomal DNA at low frequency was used to analyze the 189 isolates as well as the 53 biochemically and source-defined isolates. Approximately 3-5 ug of chromosomal DNA was used for each digest. Digestion was carried out according to supplier's specifications. Prior to electrophoresis, 5 ul of SB buffer (25% sucrose, 5mM sodium acetate, 0.05% bromphenol blue, 0.1% sodium dodecyl sulfate) was added to the digested DNA sample and mixed.

Gel Electrophoresis and Photography:

After restriction endonuclease digestion of the streptococcal chromosomal DNA, the samples were run horizontally on 0.7% agarose (Seakem ME agarose, FMC Corp) gels (0.6cm x 15cm

x 20cm) in 1xTAE buffer (50 mM Tris, 20 mM sodium acetate, 2mM EDTA) overnight at 27V. The gels were then stained for 1 hour in a solution of ethidium bromide (1ug/ml) and 1xTAE. They were then destained in distilled water for 2 hours and photographed under filtered UV-illumination (filters: red 25#A and yellow #Y48-Y2) on polaroid P/N type 665 film.

Southern Blot Assay:

The agarose gels were subsequently prepared for the transfer of the digested DNA patterns onto a nitrocellulose membrane. Firstly they were soaked in 1L of 1.5 NaOH for 1h at room temperature (RT) with constant shaking (40 rpm). Secondly they were soaked in 1L 0.5M Tris-hydrochloride (pH 7.0) for 1h and finally in 1L 3M NaCl for 3h at RT with constant shaking. The transfer apparatus was set up according to the method of Southern (12). The blots were dried at 80 °C for 2h *in vacuo* and sealed in plastic wrap and aluminum foil until required.

Preparation of 32P-Labelled DNA Probes:

The total cellular DNA of streptococcal isolates 1397, 1377 and the plasmid DNA of *E. coli* strain 807 were labelled by nick translation (10) with a few modifications (3). Approximately 1 ug of duplex DNA was labelled in one reaction. The radioactive nucleotides used were (α-^{32}P) dTTP and (α-^{32}P) dCTP (410 Ci/mol; Amersham Radiochemicals, Oakville, Ontario) with 0.1 ug/ml of DNase I (Miles Scientific Rexdale, Ontario). The reaction mixture was incubated at 16 °C for 3 hours and stopped with the addition of 0.5 M EDTA, pH 8.0. The mixture was then loaded on a Sephadex G-75 column (10ml) in 1mM Tris hydrochloride

(pH8.0)-0.25 mM EDTA and eluted. The fractions were measured in cpm and the initial peak fractions were pooled. The specific activities obtained were usually between 1.0×10^8 to 1.5×10^8 cpm/ug of DNA. All labelled probes were used within 1 month of preparation and 10^7 cpm was the average used for each hybridization reaction.

Hybridization of 32P-Labelled Probes to Southern Blots:

Southern blots were hybridized with the labelled probe according to the methods previously described (3) with some modifications. The blotted membranes were first subjected to a preannealing treatment (2xSSC, 50% formamide, 200ug/ml of yeast tRNA, 19 ug/ml of denatured salmon sperm DNA, 1x Denhardt's solution, 5% SDS) for 4 hrs. at 42°C. Before hybridization the (α-^{32}P) labelled DNA probe was boiled for 10 min. Approximately 10^8 cpm of DNA was used per preparation of hybridization buffer (same as prehybridization buffer except that it contained 0.01 M EDTA) to carry out the hybridization. The blots and probe were allowed to anneal in the hybridization mixture overnight at 42°C. The blots were then washed in successive baths of i) 2xSSC (1L) for 1 hr. at RT with shaking, ii) 0.1x SSC, 0.1% SDS (1L) for 30 min. at 50°C, iii) 2 washes (500 mls each) with 0.1xSSC, 0.25% SDS for 1 min. at RT with shaking and iv) 4 washes (250 mls each) 0.1xSSC for 5 min. each at RT with shaking. Membranes were then wet mounted at RT and exposed at -70°C to Kodak RP-Royal X-Omat film in the presence of Dupont Cronex Lightening Plus Intensifying screens. The exposure times were standardized according to the intensity of the self-annealing test control reactions.

RESULTS

In order to establish a data base for the determination of the source contamination in water, 53 strains of biochemically defined fecal streptococci of known animal source had their DNA extracted and subjected to REA using Bam HI.

The identification of the isolates within each group and the number in the group along with the percent of the total 189 strains each group represented is presented in Table 2. The REA permitted 135 of 189 or 71.5% of the strains to be grouped into 12 groups containing at least two strains of the same pattern. The results indicated that all strains from different hosts had a different REA pattern, thus establishing the uniqueness of host and fecal streptococcal strain carried. In a particular case, isolate 1346 (from the same muskrat as isolates 1383-1386) was classified biochemically as S. faecium var casselflavis while the others were classified as S. bovis (Table 1). Yet 1346 had the same REA pattern as the other strains. Furthermore, 1346 classified under the same biochemical classification as 1351, had a markedly different pattern. Thus genotypically identical organisms were being classified as phenotypically different which presented a serious problem when biochemical tests were the sole criteria on which source designation was made.

Isolates 1386, 1390 and 1395 exhibited a property called Restriction Fragment Length Polymorphism (RFLP) in their REA patterns. This was when the REA patterns were very similar yet a few discernable band differences were apparent. This indicated that although the strains are different, they are very closely related and could be linked by source as these strains were

isolated from the same source. After the REA was completed the 53 defined isolates were analyzed for potential goose and gull specific DNA probes to be used in the Southern blot assays. The 1377 probe was a S. faecium isolated from a gull source and the 1397 probe, also a S. faecium, was derived from goose feces. The 807 probe was a recombinant probe containing a Bam HI fragment of 6.6 Kbp isolated from S. durans of goose source. The human probe 1410 was of fecal streptococcal origin, representative of a group of 35 such isolates from non-laboratory workers. The other human probe 1370 was of S. faecium origin.

In the hybridization assays each probe was hybridized against itself as a positive control representing 100% homology. On the basis of hybridization reactions, a 80% to 100% reactivity as compared to the control was used to determine common host-source fecal streptococcal isolates. A reactivity of less than 40% indicated a taxonomic homologous relationship based on common ribosomal genes common to organisms within a genus. Isolates with 40% to 80% reactivity were further analysed for REA-RFLPs.

A summary of the probe results are presented in Table 2 and 3. A total of 43 of 189 or 22.8% reacted with the human probes 1410 and 1370 and the gull probe 1377. A total of 30 of 189 or 15.9% reacted with the goose probe 1397. Of the 189 strains collected, seven REA groups reacted with the human probes 1410 and 1370 as well as the gull probe and the goose probes 1397 and 1807. Probes 1410, 1370 and 1377 reacted with identical REA groups O,Q,S,V,DD,GG, and LL; probe 1397 with groups E,L,W,MM and PP and probe 807 reacted with groups D,D',and LL (Table 3). The

fact that groups O,Q,S,V,DD,GG and LL reacted with the probes from derived humans and gulls, indicated that these were most likely derived from human origin. Groups E,L, and W were most likely of goose origin and similarly family D and D' were of goose origin.

DISCUSSION

Over the years the pollution of the waters at Toronto area beaches has resulted in their closure during summer months. In order to remedy the problem each pollution source must be specifically defined. Is this caused by man or due to some element in the environment ? Toronto has combination storm sanitary sewers that empty directly into the lakes when there is heavy runoff. There are also cases of illegally connected sanitary lines to storm lines. Thus an inadequate waste-water management system results in the pollution from human sources. There is also a significantly large aquatic bird population at the water front resulting in the deposition of faeces as a non-human source of pollution. Application of methods to rectify these problems are dependent on the source. In the case of the former, the sewer system would have to be upgraded and in the case of the latter, population control measures would have to be applied to the aquatic bird population.

In previous studies we have shown the feasibility of using REA of total cellular DNA (DNA fingerprinting or genotyping) to show relatedness of a collection of organisms and thus their most probable source (2,5,6,7,8). The relationship of organisms can be further defined by the use of DNA-DNA hybridization techniques which combine DNA transfers to nitrocellulose membranes and

probing with isotopically-labelled, total-cellular DNA from a defined source or a defined recombinant DNA probe. In order to establish the suspected source (human, goose, gull, or unknown) of a contaminanting fecal organism, we have analyzed a collection of several hundred fecal streptococci using these methods.

In order to enumerate the host sources of fecal contamination in water drainage systems, reliability and specificity must exist in the testing system. Biochemical testing is a method of classifying bacteria in terms of their phenotype. With the advent of powerful molecular genetic techniques, it has become more apparent that bacterial phenotypes are dependent of gene regulatory systems that can be effected by variable cultural conditions resulting in changeable phenotypes.

Genotyping bacteria is the method proposed by Bradbury et al (5,6) as a more reliable and specific method for classifying fecal streptococcal organisms. Since the genome remains relatively more constant through natural environments, it is easier to follow the organisms as they pass through the ecological cycle (from humans to water to animals (birds) via the fecal-oral route). The overall bacterial profile, however, may change from time to time and between different geographic locations.

The overall pattern of reactions of the probe (two goose, one gull and two human) with the 44 REA groupings (A-QQ) of the fecal streptococci indicated that the goose and human probes were host specific. By contrast, the so-called gull probe 1377 reacted identically with both human probes which indicated that

the gull isolate, from which the probe was derived was in fact a human fecal streptococcal contaminant. This suggested that generalized human fecal pollution, caused by an over-used and aging sewage drainage system, has provided for an ecological transmission route through the bird populations.

In summary, the use of the total cellular DNA extracted from fecal streptococci has revealed that the strains carried by each host are unique (no two or more hosts harboured the same strain). A discrepancy with the biochemical data was revealed, as strains were classified as different species yet had the same REA pattern. This revealed the power of genotypic analysis over that of the phenotype. Commonality within a group was proven by the observation that the REA patterns of the strains from the same host were very often identical or highly similar with a few RFLP's. Strains isolated from different hosts that are biochemically identified as the same species have clearly different patterns which supported the hypothesis that differences in REA patterns without the group were greater than those differences within the grouping.

In conclusion, this study has established the use of REA to determine the most probable sources of fecal streptococci pollution. As the number of strains of known origin increases through the continuation of these studies, the expanded data base will improve the reliability of the procedure to determine the ultimate source of most of the prevailing bacterial pollution.

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TABLE 1: Biochemical and source-defined isolates of fecal streptococci are illustrated here according to their source and biochemical classification grouping. The strain code numbers are 1346 to 1405 inclusive with only the last two digits shown here. The Bam HI REA patterns of all isolates within a biochemical and source group were compared.

	Host Source						
Biochemical definition	Goose	Gull	Human	Pig	Muskrat	Duck	Dog
S. faecium		47, 74-78, 73, 79 (1)	54, 69-72, 61 (2)	55 (3)			
S. faecium var. *casselflavis*	96-04 (4)	50 (5)			46 (6)	51 (7)	
S. bovis					57 81-86 (8)		60 (9)
S. faecalis var. *zymogenes*						93-95 (10)	
S. faecalis var. *liquefaciens*	59 (11)					58 (12)	
S. durans				48 (13)		53 87-92 (14)	52 (15)
S. faecalis var. *faecalis*	49 (16)						

TABLE 2: REA groupings and their relationship to the probes.

REA Group	Isolates Within Group	No. In Group	% Of Total	80-100% Homology With Probe				
				1410 Human	1370 Human	1377 Gull	1397 Goose	807 Goose
A	2,4,6,9,10,11,14	7	3.7					
B	1,3,5,7,8,13,16,17, 18,19,20,21,22,23,24	15	7.9					
B'	25	1	0.6					
C	12,15	2	1.1					
D	26,27,29,31,32,33,34, 35,36,37,38,39,40,42, 43,45,46,47,50,51,52, 53,30,55	24	12.7					X
D'	28,41,44,48,49,54	6	3.2					X
E	56,57,58,59,60,61,62, 63,64,65,66,68,69,70, 72,73,75,76,77,79,80, 81,82,83,84,85,86	27	14.3				X	
F	78,93,94,105,106,109, 110,104	8	4.2					
G	87	1	-					
H	88,96,97,102,107,182	6	3.2					
I	135	1	-					
J	95	1	-					
K	98	1	-					
L	99	1	-				X	
M	100	1	-					
N	101	1	-					
O	118,119,120,121,124, 125,126,127,128,129, 130,131,132,133,134, 138,139,140,141,142, 144,145,146,159,160, 163	27	14.3	X	X	X		
P	136	1	-					
Q	137	1	-	X	X	X		

TABLE 2: (CONT'D).

REA Group	Isolates Within Group	No. In Group	% Of Total	80-100% Homology With Probe				
				1410 Human	1370 Human	1377 Gull	1397 Goose	807 Goose
R	143	1	-					
S	183,185,186,187,188, 189,169,171,173,180	10	5.3	X	X	X		
T	184,174,175,176,181	5	2.6					
U	177,178,179,156A	4	2.1					
V	170,171	2	1.1	X	X	X		
W	164	1	-				X	
X	165	1	-					
Y	166	1	-					
Z	167	1	-					
AA	169	1	-					
BB	172	1	-					
CC	182	1	-					
DD	147	1	-	X	X	X		
EE	155	1	-					
FF	154	1	-					
GG	153	1	-	X	X	X		
HH	152	1	-					
II	151	1	-					
JJ	150	1	-					
KK	149	1	-					
LL	148	1	-	X	X	X		X
MM	156B	1	-				X	
NN	157	1	-					
OO	158	1	-					
PP	161	1	-				X	
QQ	162	1	-					

TABLE 3: Summary of the Southern blot assays as detailed in Table 2.

Probe Used	Groups Giving 80-100% Homology
1410 Human	O,Q,S,V,DD,GG,LL
1370 Human	O,Q,S,V,DD,GG,LL
1377 Gull (S. faecium)	O,Q,S,V,DD,GG,,LL
1397 Goose (S. faecium var. casselflavis)	E,L,W,MM,PP
807 Goose (S. durans)	D,D',LL

NOTE: The "gull" probe also hybridized with human S. faecium isolates.

LIQUID AND SOLID SAMPLE INTRODUCTION FOR INDUCTIVELY COUPLED PLASMA ATOMIC EMISSION AND MASS SPECTROMETRY.

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1. Liquid Sample Introduction for Inductively Coupled Plasma Mass Spectrometry

1.1 Background.

Inductively Coupled Plasma (ICP) Mass Spectrometry (MS) has demonstrated that it can provide detection limits at least one to two orders of magnitude better than those which can be obtained with icp atomic emission spectrometry (AES). ICP-AES is superior to furnace atomic absorption in several important regards: (1) it appears to be less susceptible to matrix effects and (2) it is a multielement technique. One of the advantages offered by furnace atomic absorption is the ability to analyze microsamples. One of the most serious continuing problems with icp-ms is the spectral problems caused by oxide formations of various isotopes. In previous reports we have discussed the direct sample insertion device (DSID) and its application for icp-ms. The device has been installed at the Ministry of the Environment and is in regular operation. The technique involves placing a liquid sample on a wire loop. The loop is moved underneath the plasma where it is dried in approximately 30 s. The wire loop with sample is then inserted rapidly into the plasma. The sample is vaporized rapidly and produces momentarily a large concentration of the analyte in the plasma.

1.2 Spectral Simplification.

Because the sample is dry (water removed) when using the DSID for icp-ms, the resulting mass spectrum has drastically reduced oxygen interferences. Recent experiments with trace level determinations of Fe have demonstrated an order of magnitude improvement in the detection limit when determining Fe (mass 56) in the presence of real samples which containing significant amounts of Ca (mass 40) because CaO (mass 56) interferes. Even though it would seem to be possible to do a spectral strip known the Ca:CaO ratio, the noise in the signal precludes the determination of Fe at low levels when the Ca level is high (i.e. the residual Fe signal is buried in the CaO noise).

1.3 Expert System.

We have developed an expert system operating on an IBM-PC. The system is designed to perform one of several tasks. In one form it can be used to operate a sequential scanning spectrometer for icp-aes. By an "expert" choice of lines it can compensate for spectral overlaps. In an icp-ms form, it can predict spectral overlaps (such as those produced by oxygen) and "strip" or compensate for spectral overlaps.

1.4 Precision Improvement and Automation.

Experiments have demonstrated that the wire loop sample introduction system is capable of producing highly reproducible results. With an automated sample applicator relative standard deviations of less than 1% are possible. The development of a robotic sampler is under investigation. The system would provide considerably more than automated sample application. Based on an initial measurement, the system would use expert knowledge to perform a matrix matching experiment. A new, matrix matched sample would be injected into the instrument until a sufficiently close match had been made, based on the accuracy requirements of the experiment.

Other experiments have demonstrated that wires can be replaced manually with less than a 10% loss in accuracy using hand made wires. Since the lifetime of the wires is quite long, and the calibration time is relatively short, it is probably not necessary to automate the replacement of wire loops.

2. Inductively Coupled Plasma Atomic Emission Spectrometry

2.1 Background. We have previously reported on the use of the the DSID for icp-aes using a single channel detection system with photomultiplier detection and a multichannel photodiode array based system. This initial research clearly demonstrated the need for simultaneous multichannel detection with real time background correction. The reasons can be briefly summarized:

1. The DSID, as well as a number of other promising sample introduction systems, produce transient signals in the plasma. The duration of the signal is sufficient short (0.3 s in the extreme) that scanning systems can not acquire all the information while it is available.

2. Real samples can provide complex changes in the spectrum in the spectral region of the analyte. Due to the short residence time of the analyte and any interfering species, background spectral information must be rapidly acquired so as to provide sufficient information to allow accurate correction data.

2.2 Thought Pattern.

The primary problem with solid samples is the time required to do a digestion. Secondary problems include reagent contamination and dilution. The DSID offers one possible choice, however, it (and other techniques like it) obviously presents a serious problem at the detection end of the instrument. Slurries offer the potential for the direct analysis of solids using conventional detection systems, since the signal is relatively static for sufficient time to provide background correction. If the concentrations can be kept sufficiently high in slurries, then the detection limits could match those of techniques like the DSID. On the other hand, why not simply reduce the sample handling time by automation of the chemistry involved. Below I will discuss some of our work and thoughts in each of these areas

2.3 Detection System Modifications.

There are several methods of background correction in use today on multichannel spectrometers. They usually involve shifting the entrance slit or moving a refractor plate inside the spectrometer. The effect of both of these is illustrated in Figures 1, 2 and 3 below. As Figure 1 indicates, anything that changes the incoming angle of the radiation to the grating will cause a shift of the spectrum. The moving entrance slit has the unfortunate byproduct that it obtains its' spectral shift by looking at a slightly different portion of the plasma (Figure 2). This probably makes little difference when using liquid sample introduction, because the sample is distributed relatively uniformly over the "hole" which has been punched in the plasma. New transient methods such as the DSID can not be expected to uniformly distribute the analyte, consequently we expect that a moving slit background correction scheme will not be adequate for solid sample transient work. Figure 3 illustrates the galvanically driven refractor plate scheme used in the Thermo-Jarrell Ash line of multichannel spectrometers. While it may cause some minor problems (such as shifts in stray light levels due to increased reflectance from the inner surface) we expect it to be superior. It offers the additional important advantage of speed. The manufacturer reports that it can settle in as little as 3 ms. Unfortunately, the Jarrell-Ash family now uses a controller computer inside the direct reading spectrometer.. The controller's program resides in ROM and strictly limits the types of instructions that can be obeyed by the controller. The software/hardware seems to be excellent for traditional signal processing, but totally unsatisfactory for transients. We are obtaining the controller codes from Jarrell-Ash and hope to able to report success with the direct control of the system, rather than using the "canned" Jarrell Ash software.

2.4 Slurries

We have obtained several different nebulizers which should be capable of slurry nebulization. These include the Legere nebulizer and the Jarrell Ash high solids nebulizer. We intend to evaluate several unusual modifications.

2.4.1. Temperature

We intend to heat the slurry to approximately 95 degrees centigrade. The argon gas will be heated to approximately 300 degrees. The spray will go into a heated spray chamber. The result should be to reduce the droplet size drastically. The resulting aerosol stream will then be run through a condenser to remove as much water vapor as possible. This should allow us to input a much larger effective percentage of solid.

2.4.2 Plasma Composition

We believe that there may be considerable advantage to changing the plasma composition by introducing either small amounts of oxygen into the "plasma" gas stream or large amounts into the "aerosol" stream. Either one or both may result in a much faster breakdown of the particles. The effect should be to allow a much larger particle size to be introduced. This will allow a much shorter sample grinding stage, possibly as short as 60 seconds.

2.5. Stream Digestion

We have begun an investigation into the possibilities of stream (flow) digestion systems for solids. This would involve a high pressure pump which would pick up the solid as a powder and combine it with appropriate chemicals, usually mineral acids and pump the mixture through a microwave oven. Our initial experiments demonstrate that thin tubes filled with water can heat very rapidly. With a restricted orifice at the end of the tube high pressures and temperatures should be obtained. The effectiveness of microwave digestion in relatively conventional bombs has already been demonstrated. We expect that the digested sample will then flow directly to the ICP after a de-aeration step to remove gases. We will report on the progress with this system.

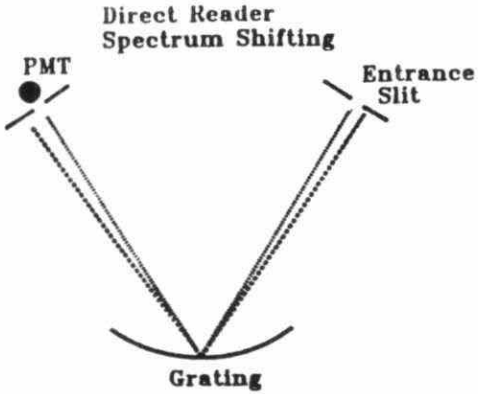

Figure 1

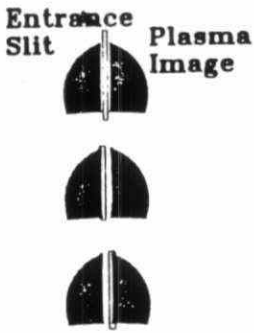

Figure 2

Moving Entrance
Slit Spectrum
Shifting

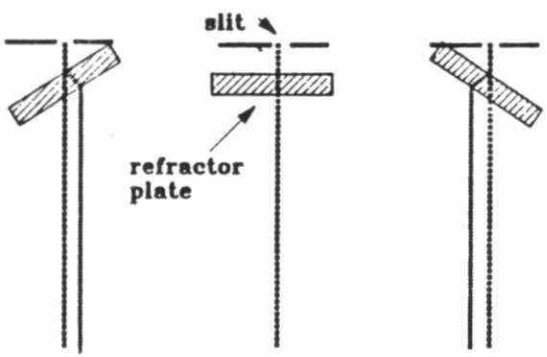

Figure 3

Refractor Plate
Spectrum Shifting
Technique

Technology Transfer Conference

Ontario Ministry of the Environment

Screening Methods for Air and Water Samples by ICPMS

by

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This submission describes investigations in 2 main areas of inductively coupled plasma mass spectrometry (ICPMS) research.

These are:

(1) Electrothermal vapourizer (ETV) Sample Introduction.

(2) Gas chromatography with an ICPMS detector.

The ICPMS chromatography detector, developed in our laboratory, is a new type of mass spectrometry detector for chromatography.

[A] ELECTROTHERMAL VAPOURIZER (ETV)

Plasma source mass spectrometry, using solution nebulization, is capable of excellent powers of detection. In many cases detection limits are in the pg/ml range, comparable to or better than Furnace atomic absorption and neutron activation. However, many environmental samples have important elements at levels which, after sample preparation and dilution, are below nebulizer ICPMS detection limits.

We have been developing an electrothermal device for sample introduction into a plasma (1). Work, with an Re metal Filament ETV, reported here last year, suggested that for many elements a ten fold improvement in detection limit should be achievable. Indeed such was obtained for Cu and Pb (2). The Re filament could be heated to a maximun temperature of 1800°C. At this point appreciable Re is volatilized and causes plasma loading (or ionization suppression) with a resultant loss of analyte signal.

A graphite boat filament ETV is under development to be used for the many elements requiring a higher vapourization temperature. With this device early results showed unacceptable shot-to-shot reproducibility. It has been demonstrated that the flow in the transfer line is laminar (i.e. Reynold number is <<2000) so flows in this section should not be to blame.

Park and Hall (3), using an isotope dilution approach have reported excellent results. They obtain detection limits which are generally 10 times better than nebulizer values. The Park and Hall approach (isotope dilution) requires an extensive library of expensive isotopes. Also several important elements are monoisotopic. Thus we decided to persist with our more conventional approach. To this end and in this years work, we made substantial improvements in both the ETV cell design and in the power supply to the ETV.

It is important to remember that the ETV approach has several potential advantages over the nebulizer method:

(1) Better detection limits.

(2) Can be used with samples of limited volume.

(3) Drying and Ashing can help reduce ionization suppression and spectral overlap interferences.

EXPERIMENTAL

There was a tendancy with the old ETV design (up to summer, 1987) for the swriling gas flow inside the ETV to collapse onto the filament. This resulted in contamination of the filament posts and poor reproducibility of the signal.

The following must occur if the ETV operation is to be satisfactory. The gas bubble (aerosol sample) produced by the hot filament must rise steadily and smoothly within the swirling gas flow. Condensation of the hot aerosol into a microparticulate (< 1μ) must be complete before the bubble reaches the upper walls of the glass dome. Then the swirling stream of argon (introduced tangentially) will entrain the cooled aerosol and carry it into the plasma without large losses. If the bubble collapses back onto the filament and/or the aerosol does not cool before reaching the upper glass dome walls, poor sensitivity and poor repeatability are likely.

A new ETV design has been created and the most significantly improved features of this device are:

(1) Air foils are placed midway between the glass dome wall and the electrode assembly.

(2) The new glass dome has a tall chimney extending upwards from the region of the electrodes.

(3) The radius of curvature of the lower curve in the region of air foil (r_2) was made to be very much less than r_1, the radius of curvature of the curve at the base of the chimney.

(4) The electrode assembly was streamlined to better fit with the geometry of the glass dome.

These design features, shown in the photograph, lead to an acceleration of the gases in the electrode region thus minimizing disturbance of the flow and preventing the hot bubble of aerosol from collapsing onto the electrode assembly.

Additional features of the new design are as follows:

(1) Provision has been made for the introduction of a reactive gas, at low flow rate over the filament.

(2) The electrode posts may now be water cooled.

It was demonstrated by Kirkbright (3), in work with an ETV and ICPAES, that introduction of a halogen containing gas, such as freon, improved the signal characteristics of the less volatile elements (e.g. V, W, Ti). The halogen in the freon reacts with many metals forming a more volatile metal halide. As a result the signal peak shape is sharper and occurs earlier in the vapourize cycle.

Cooling of Electrode Posts

Uncooled electrode posts were found to be satisfactary in work using the thin Re metal filament because of its low mass and high conductivity. Cooling to near room temperature was rapid. Time between sample volatilizations was short.

The graphite boat filament is much larger and graphite has poorer heat conductivity. Using operating conditions similar to those employed for the Re filament, very poor signal reproducibility is obtained. At the beginning of a run the first sample injection (2 ul) onto the graphite filament formed a nice spherical droplet in the center of the sample introduction well. However, after a few firings the sample droplet soaked into the boat surface, spreading out along its length. This problem became worse as the sample run goes on. In the extreme, the sample may spread out so much that it contacts and contaminates the electrode posts. As a result poor shot-to-shot reproducibility is obtained and a memory effect occurs as the electrode posts become appreciably contaminated.

Treatment between firings with 100 ul of ethyl alcohol resulted in quicker cooling and better droplet shape. However, the addition of this solvent is a potential soure of contamination and was for this reason discontinued.

It is possible to modify the electrode assembly to permit water cooling. To this end a water flow was introduced to contact the base of each electrode post. Using this design modification reproducible sample injections were obtained.

Carrier Gas Flow Rate

With the new ETV design and introduction of a graphite boat filament it was essential to reinvestigate the signal characteristics with changes in carrier gas flow rate. A flow rate of between 3.0 and 3.5 l/min was found to be optimal. This compared to 2.0 l/min using an Re filament with the previous equipment (1).

ETV Power Supply Modifications

With the previous system, voltage to the electrodes was varied manually. In the quest to minimize shot-to-shot analyte signal differences an automated voltage controller was designed and built.

The circuit diagrams, though available, are not included here. The main features of the supply are:

(1) It has 3 heating cycles.

(2) Continuous variation in final temperature of each cycle is possible.

(3) Continuous variation in the temperature rise rate can be obtained.

(4) The output voltage range to the filament can be varied to suit either metal or graphite filaments.

RESULTS

The instrument conditions used for most of this work were (these are compromise conditions)

Plasma Forward Power 1.0 - 1.1 kw

Reflected Power near 0W

Gas Flows to Torch

Coolant 14 l/min

Auxillary 0.7 l/min

Carrier 2 l/min

Resolution is about 1 amu at 10% peak height.

We have tested the graphite filament for several of the difficult to determine (relatively non-volatile and complex solution chemistry) elements. The following for Sn is typical of results for these "difficult" elements.

Tin

Tin is a particularly troublesome element to determine because of its tendency to form "oxy" compounds in solution. It also has volatile halide compounds and yet in other forms is relatively refractory. There are difficulties in achieving complete dissolution, of Sn containing samples, when using the "usual" techniques. Hydrofluoric acid may be essential in the decomposition mixture even when Sn is not bound up in silicates. Additionally relatively poor detection limits are obtained for Sn by furnace atomic absorption and plasma emission spectrometries.

Because of the tendency of some of the Sn present to form halides during the decomposition step, it is essential, to add a matrix modifer so that no Sn losses

will occur from the ETV filament during drying and ashing steps (whch come prior to the volatilization step). Work in our laboratory, relating to ETV volatilization and furnace atomic absorption Sn determinations, showed that addition of Pd, under reducing conditions, prevented Sn loss up to 1400°C. A variety of reducing agents were tested for this purpose with hydroxylamine hydrocholoride giving the best results.

In interference free solutions the detection limit for Sn by ETV/ICPMS is about 0.006 ng/ml. The following (Table (1)) is a compilation of results for the determination of Sn in some Standard Reference Samples.

Table (1) Sn (^{120}Sn) in Standard Reference Samples

Sample	Amount (ug/g)
NRC Tort - 1	0.130 ± 0.050
	(0.135)
NBS Bovine Liver	0.220 ± 0.070
	0.230
NBS River Sediment	3.9 ± 0.6
	(3.7)
NBS Marine Sediment	
(a) BCSS - 1	2.0 ± 0.4
	(1.9)
(b) MESS - 1	4.0 ± 0.6
	(3.9)

* ( ) are accepted values

The mean values are generally in suitable agreement with the Accepted Values. The precision is still poorer than should be expected (In this regard the ETV is undergoing further modification-see section on P.8).

Another difficulty was the tendency for Sn to contaminate the ETV. For example, after running 2 ul of 100 ppb Sn (signal about 100,000 cts/s) the blank (2 ul) of 5% nitric acid had risen from 30 cts/s to about 4500 cts/s.

The following 2 elements also illustrate important points of difficulty. These problems will be found to be typical of other related elements.

Vanadium

V is very difficult to volatilize from an ETV filament and was found to contaminate both the graphite boat filament (at the cool ends) and the glass dome. Signals for V are typically long and flat rather than having sharp peaks. Early results using the new ETV design, together with freon, suggest that contamination is lessened and the volatility is increased. However neither problem was completely rectified. Results for real samples were up to 50% too low both by standard addition and by direct calibration.

Gold

Au could be readily determined using the graphite filament. However if a solution (standard or simple) was run which was relatively high in Au a serious memory effect appeared.

e.g. 2 ul 100 ppb Au = > 200,000 cts/s

Blank = 20,000 cts/s

(50 cts/s before)

Inteference Effects

These were covered in detail in last years presentation. However, additional difficulties were identified and are discussed herein:

The most serious interferences identified to date for ICPMS are:

(1) Ionization suppression

(2) Spectral overlap (especially troublesome at mass 80 and below).

Both these types of interferences can sometimes be reduced by using the ETV approach to sample introduction. This is accomplished by ashing away some of the predominant acid anions and major matrix ingredients before the vapourization step. In this way matrix loading (and thus ionization suppression) of the plasma is reduced. Additionally water is removed during the drying step. This suppresses the large number of molecular species which are water derived (hydrides, oxides etc.) and hence reduces the likelihood of spectral overlap.

However volatilization interferences can be a unique and serious problem in using ETV sample introduction. The volatility of both analyte and matrix species is dependent on their forms on the filament just prior to the vapourization step.

A dramatic example is as follows:

10 ul of 100 ppb V (by itself in dilute HNO_3)

gives an elongated peak with poor peak height development. Even after 3 seconds at 2600°C the signal has not returned to base line. Signal was about 10,000 cts/s 100 ppb

10 ul of 100 ppb V + 10 ul of 10% $MgCl_2$

At 2600°C 2 peaks are obtained but the major one is very sharp with a peak width of less than 1 sec. Signal was about 50,000 cts/s

We are now investigating the possibility of using the $MgCl_2$ approach (at much less than 10%) to increase the volatility of other analyte species.

Suggestions For Our Further Work

(1) Improve the metal post to graphite filament contact. With the present system arcing occurs after 30 to 40 firings in this contact. Graphite Extensions of the electrode posts should be made on top of the metal sections. Then the graphite filament should be clamped to the graphite extensions.

(2) Remove the ball joint from the glass dome. Use a continuous glass tube from the top of the glass dome to the tygon tubing.

(3) Improve the cooling of the electrode posts by extending the copper tubing up into the center part of the posts.

(4) Try other aerodynamically suitable shapes for the glass dome.

(5) Test other metallic filaments e.g. W, Ta. Work to date suggests that filaments of only 1 composition are not optimun for all elements.

[B] ICPMS A NEW MASS SPECTROMETRY DETECTOR FOR CHROMATOGRAPHY

Last year we reported results using this detector for the gas chromatographic determination of alkyltin compounds. Separation of 3 alkyltins (Me_3SnP_3, Me_2SnPe_2, $MeSnPe_3$) was good.

Separation of Organoselenides by Packed Column GC and Detection by ICP-MS.

Separation of dimethylselenide and dimethyldiselenide was achieved using packed column GC with ICP-MS as a selenuim specific detector. The GC column contained 30% OV-1 on Chromasorb W.

Choice of carrier gas flow rate was somewhat restricted since too high a gas flow tended to blow out the plasma. A flow of 80 ml/min of Argon was chosen. This was not significantly varied during the period of work reported here.

Oxygen was added to the carrier gas flow in the transfer line between the GC and the ICP torch in order to bring about more complete combustion of organic matter in the plasma. The 0_2 flow rate was 20 ml/min. Lower flow was not sufficient and higher flow tended to extinguish the plasma.

Summary of chromatographic conditions:

Argon carrier gas flow rate: 80 ml/min

Oxygen flow rate: 20 ml/min

Injector temperature: 200°C

Transfer line temperature: about 200°C

Temperature program: 80°C isothermal

to elution of first peak followed by rise in temp. to 180°C at the maximum ramp speed.

Plasma forward power was varied to study the effect of plasma power on the magnitude of the solvent peak. This peak occurs between those of the two sample components. A forward power of 1.15 kW was found to minimize the toluene peak.

Sample volumes of 0.5 ul to 7.5 ul were successfully injected and diluted without extinguishing the plasma. No larger injections were attempted.

Further work remains to be done in several areas:

1. Make a careful measurement of retention times. Approximate values for dimethylselenide and dimethyl diselenide were 40 and 67 seconds respectively.
2. Study the effect on the signal of variations in the transfer line temperature.
3. Do a more careful determination of detection limit and study the effect on the signal of injecting larger aliquots of more dilute standard solutions. Present values are adversly affected in that ^{80}Se, the most sensitive isotope is isobaric with $^{80}Ar_2$. Thus ^{78}Se was used.
4. Progress to do "real" samples and other elements.

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ADVANCED MASS SPECTROMETRIC TECHNIQUES FOR THE IDENTIFICATION OF UNKNOWN ORGANIC COMPOUNDS

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Classical techniques used in the identification of unknown organic compounds have included fractionation and purification procedures, determination of physical constants, tests for specific elements, classification by solubility and acid-base character, tests for compound classes and preparation of derivatives. These analytical techniques required relatively large amounts of a pure compound. With the advent of instrumental techniques such as infrared spectroscopy (IR), ultraviolet spectroscopy (UV), nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and X-ray diffraction, complementary information could be obtained on smaller amounts of a pure compound. Subsequent advances have incorporated the fourier transform principles into spectrometric techniques resulting in FT-IR, FT-NMR and FT-MS with their higher inherent sensitivities.

Environmental organic analysis requires very sensitive techniques that can deal with mixtures of components present at trace levels. These separation/detection requirements have been met by the combined techniques of gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) and, more recently, gas chromatography/fourier transform

mass spectrometry (GC/FT-MS), gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS) and gas chromatography/fourier transform infrared spectroscopy/mass spectrometry (GC/FT-IR/MS) [1-3].

At the present time, GC/MS is the most technologically mature and the most widely used of these instrumental techniques. Identification of trace amounts of organic components in mixtures by GC/MS is the norm because techniques like FT-IR oftentimes cannot meet the sensitivity requirements of the analysis.

In a typical GC/MS run, a mass spectrum is obtained for each component that is chromatographically separated by the GC. Each mass spectrum is then matched against a reference library of mass spectra. Libraries can range from 42,000 [4] up to approximately 123,000 [5] entries. This technique of library searching is limited by the number of entries in the library and the capacity of the computer to assimilate and search larger libraries [6]. Criteria such as purity, fit and reverse fit are used to evaluate the validity of the match. Because of the large number of possible organic compounds that can be encountered in the environment, a valid library search cannot always be found. The mass spectra of unknowns must then be interpreted from first principles.

Advanced mass spectrometric techniques that can aid this interpretation include accurate mass (empirical formulae) determinations, linked scanning and mass analysed ion kinetic energy spectrometry (MIKES) [7].

A high resolution double-focusing reversed geometry mass spectrometer has these capabilities and is pictured in Figure 1. When a magnetic and electrostatic sector are connected in series, the mass-to-charge ratio of the ion reaching the detector (electron multiplier) may be determined to four decimal places (accurate mass) while those determined by quadrupole mass analyzers are only accurate to one decimal place (unit mass resolution).

A high resolution mass spectrometer, because of its ability to resolve accurate masses, can differentiate between dioxins and interferences such as polychlorinated biphenyls (PCB). The Ministry's VG-ZAB-2F mass spectrometer is being used to routinely perform high resolution mass spectrometry (HRMS) dioxin confirmations at 12,000 resolution.

Accurate mass measurements of the major ions in a mass spectrum allow the determination of the empirical formulae of these ions. Also, structures that are consistent with the isotopic ratios can then be postulated.

Linked scans are performed by scanning the magnetic and electrostatic sectors in specified relationships to monitor metastable ions formed in the first field free region (FFR1, the region between the source and magnetic sector). This allows determinations of daughter ions from a specific parent ion (B/E), parent ions of a specific daughter ion (B^2/E) and ions which lose a specific neutral ($(B/E)(1-E)^{1/2}$). These determinations are important because several fragmentation pathways may occur simultaneously and ions of a particular mass may not necessarily

originate from an adjacent higher mass ion.

MIKES experiments are performed in the second field free region (FFR2, the field free region between the magnet and electrostatic sector). The parent ions are preselected by the magnetic sector and then are monitored by the electrostatic analyser. The structural information obtained from MIKES is similar to that obtained from B/E scans but the kinetic energy release data which provides information on the type of fragmentation reaction is retained. This type of scan is relatively free of artifacts; however, it is limited to unit mass resolution on the parent ion.

The following example shows the analysis of a fish sample that was initially extracted for dioxin determination. As shown in Figure 2, the HRMS (10,000 resolution) total ion chromatogram (TIC) of this sample gives two large peaks. The mass spectrum for scan number 213 as shown in Figure 3 was succesfully determined by library matching to be a cholesterol-type steroid.

The mass spectrum of the component observed at scan 141 is shown in Figure 4. A library search of this compound shown in Figure 5 gives a number of good matches (>800) for the forward search (mix); however, the purity and reverse fit values are poor. On visual inspection of the first three library search spectra (Figure 6) it is obvious that none of the library spectra could be that of the unknown. The good forward search match is due mainly to the m/z 65 and m/z 91 ions present in all three spectra.

Determination of the accurate mass, and therefore the corresponding molecular formula of this compound as shown in Figure 7 gave five possible formulae within 5ppm of the unknown. Separation of two compounds that differ in mass by 5ppm require 200,000 resolution. Therefore, isotopic ratios for M^+ and $(M+1)^+$ must be used to determine the most probable molecular formula. $(M+1)^+/M^+$ for the unknown was determined as 22.5%. Of the possible formulae in Figure 7, only $C_{20}H_{18}O_2$ (22.4%) matches with the unknown.

To obtain more information on the $C_{20}H_{18}O_2$ compound, the B/E linked scans for m/z 290 and m/z 91 and the MIKES for m/z 290 were determined (Figure 8). These scans show that the molecular ion (m/z 290) fragments mainly to m/z 199 ($C_{13}H_{11}O_2^+$) and m/z 91 ($C_7H_7^+$) and that m/z 65 ($C_5H_5^+$) is formed exclusively from m/z 91. The appearance of a mass spectrum is strongly dependent on the internal energy of the parent ion [8,9]; therefore, methods such as MIKES and linked scanning which determine the fragment ions of low energy parent ions (if no collision gas is present in any of the field free regions) give information complementary to normal mass spectra. In this case, the m/z 199 ion which is absent in the normal mass spectrum is a significant peak in the MIKES and linked scans.

The m/z 91 to m/z 65 reaction is strongly indicative of a benzyl moeity. Also, the simplicity of the mass spectrum indicated the unknown compound was highly symmetrical. The following structure:

was postulated and synthesized. The mass spectrum (Figure 9a) of the synthesis product (scan 147) matched that of the unknown (scan 141, Figure 9b); however, GC retention times (scan numbers) were slightly different due to the large amount of benzyl chloride present from the reaction mixture. To confirm that these compounds were identical, both the unknown and the synthesized compound were coinjected. This gave an enhancement of a peak observed at scan 143 which as seen by the mass spectrum of this scan (Figure 9c) is identical to that of both the unknown and the synthesis product. The mass spectrum of the unknown (1,4-dibenzyloxybenzene) is in good agreement with that previously reported by Danks and Hodges [10]. They also report the mass spectra of two other possible isomers, 1,2-dibenzyloxybenzene and 1,3-dibenzyloxybenzene. Both isomers contain a considerable peak (about 10%) at m/z 181. The absence of this peak in the mass spectrum of the unknown and that of 1,4-dibenzyloxybenzene supports the conclusion that the unknown compound is the 1,4- isomer.

In summary, the determination of the structure of an unknown organic compound requires the use of a variety of complementary data. This information may then be used to postulate a structure for the unknown. In order to confirm the structure of an

unknown, the mass spectra as well as other physical data of a standard (a compound of known structure which is either bought or synthesized) and the unknown must match.

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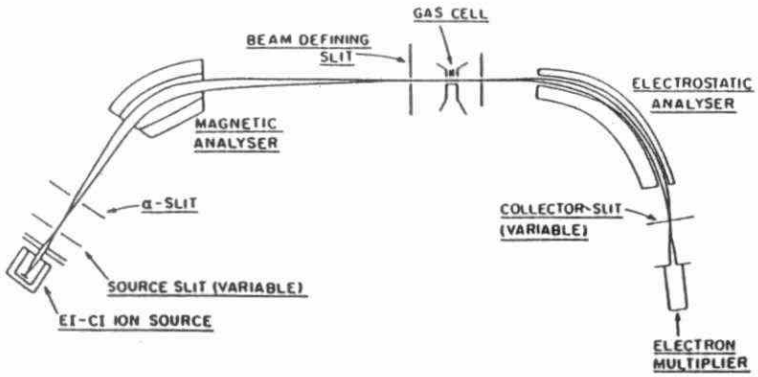

Simplified diagram of double focusing mass spectrometer of reversed geometry (VG-Analytical ZAB-2F).

FIGURE 1: A Reversed Geometry Mass Spectrometer

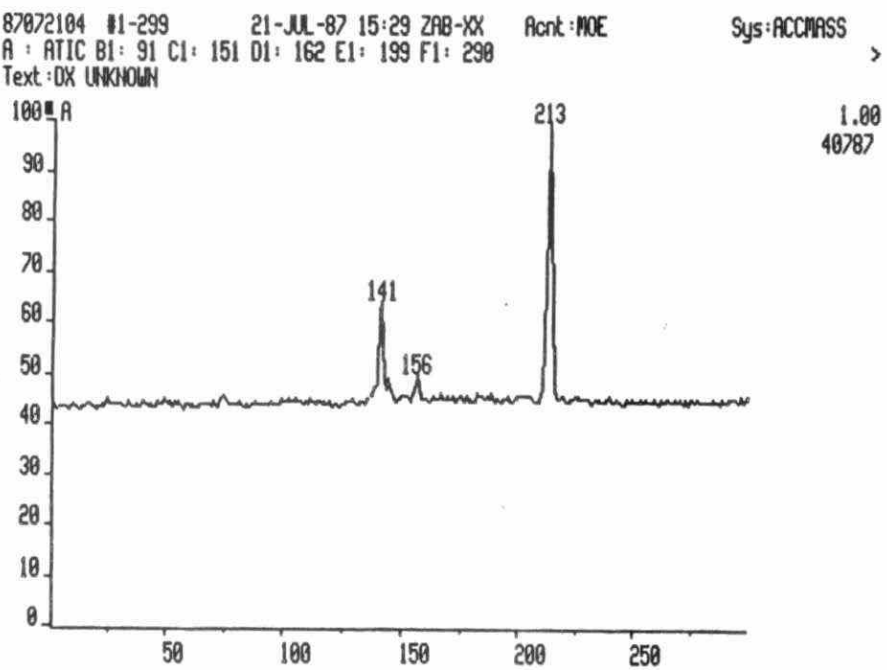

FIGURE 2: TIC of a Fish Extract

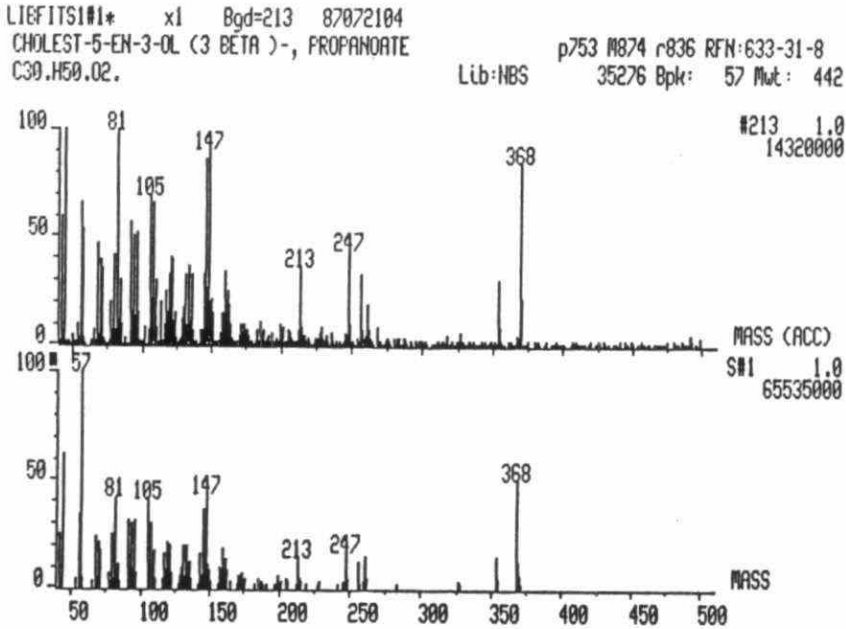

FIGURE 3: Library Match of Scan #213

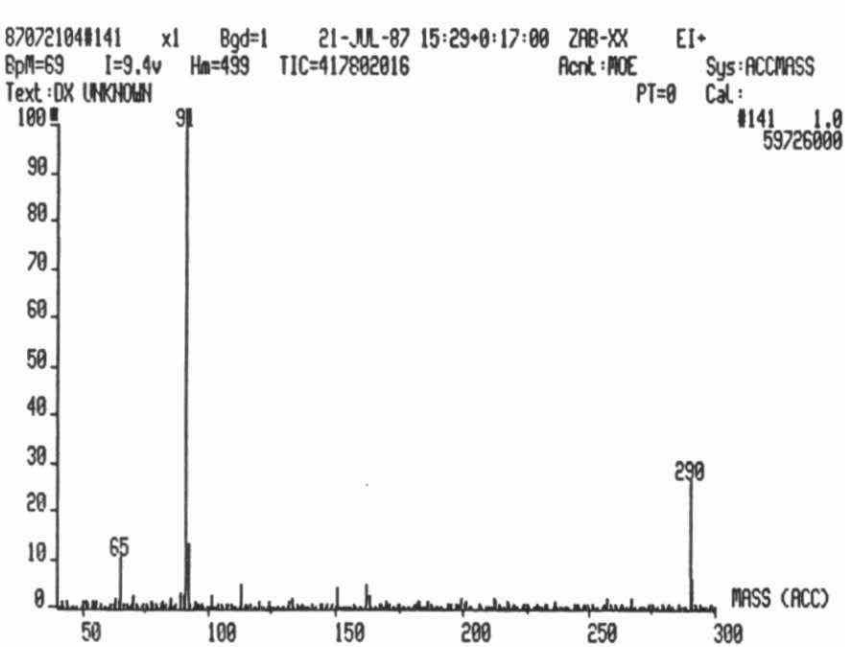

FIGURE 4: HRMS of Scan #141

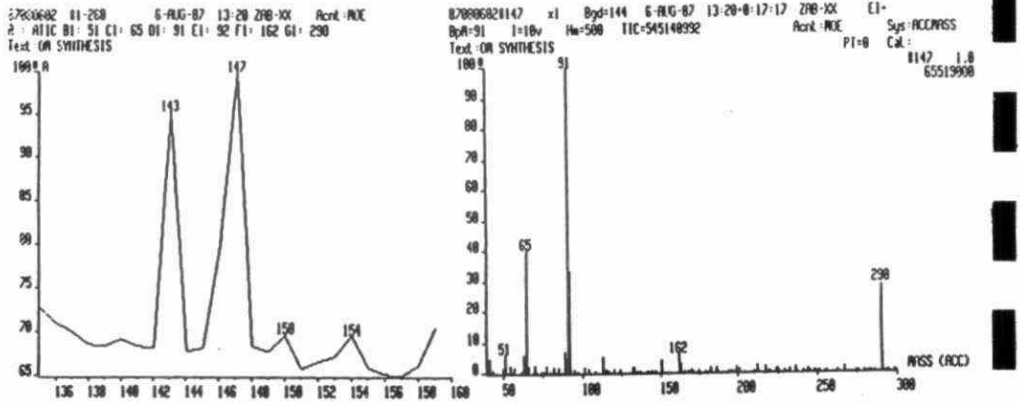

FIGURE 9a: TIC and HRMS of Synthetic Product

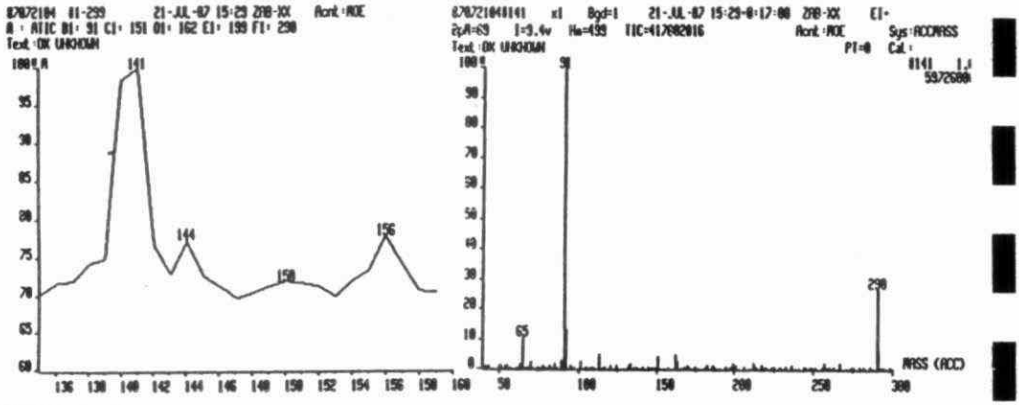

FIGURE 9b: TIC and HRMS of Unknown

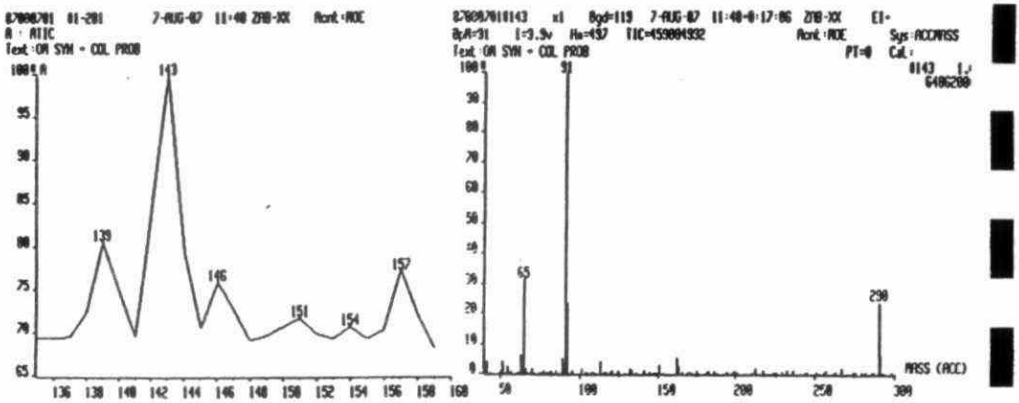

FIGURE 9c: TIC and HRMS of Coinjection

```
87072104#141    x1    Bgd=1      21-JUL-87 15:29+0:17:00  ZAB-XX      EI+
BpM=69    I=9.4v    Hm=499    TIC=417802016                Acnt:MOE        Sys:ACCMASS
Text:DX UNKNOWN                                                PT=0    Cal:
   M/E   C   H  O  N CL CL BR BR    PPM     DBE     ACC.MASS
         12                35 37 79 81
-------------------------------------------------------------------------------

  290  14  27  0  1  0  0  0  1   -0.2     1.5   290.1306400
       15  24  0  1  1  1  0  0   17.1     3.5   290.1256302
       14  24  0  2  2  0  0  0   -3.7     3.0   290.1316544
       17  21  1  1  1  0  0  0   -2.0     7.5   290.1311672
       18  16  1  3  0  0  0  0    4.3    12.5   290.1293373
       20  18  2  0  0  0  0  0   -0.3    12.0   290.1306799
       14  23  3  1  0  1  0  0  -10.7     3.5   290.1336964
       12  21  3  3  1  0  0  0   11.9     3.5   290.1271445
       14  23  4  0  1  0  0  0    7.2     3.0   290.1284871
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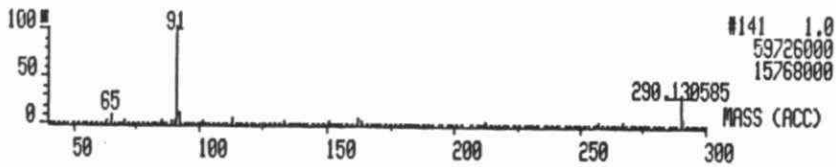

FIGURE 7a: Determination of Molecular Formulae

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87072104#141    x1    Bgd=1      21-JUL-87 15:29+0:17:00  ZAB-XX      EI+
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Text:DX UNKNOWN                                                PT=0    Cal:
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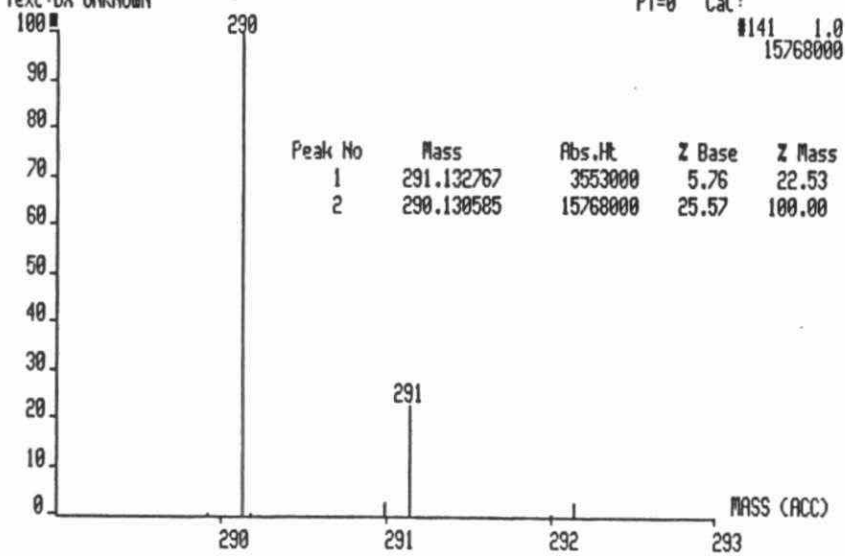

FIGURE 7b: Isotopic Ratio of Molecular Ion Cluster

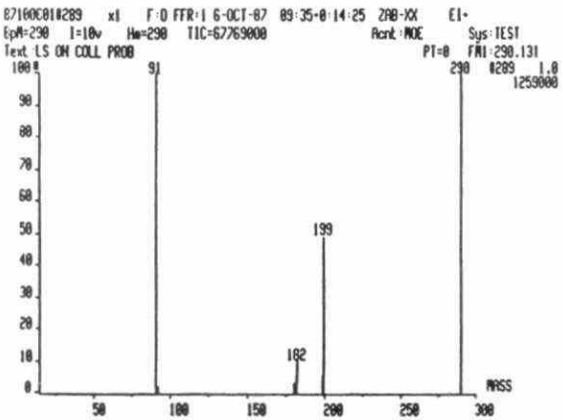

FIGURE 8a: B/E Linked Scan of m/z 290

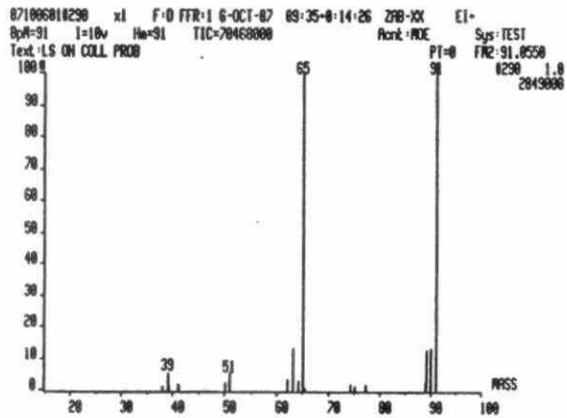

FIGURE 8b: B/E Linked Scan of m/z 91

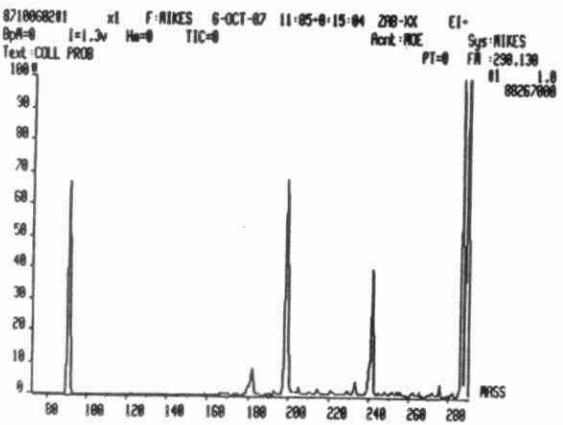

FIGURE 8c: MIKES of m/z 290

LIB Library Search Report of 87072104#141 Library:NBS 06-OCT-87

38785 Library spectra compared for BEST FIT
36 matched 4 or more of the 16 Largest peaks in the unknown

Fit	Entry	Compound Name	Reference
1	9275	BENZENE, HEXYL-	1077-16-3
2	17117	BENZENE, (4-BROMOBUTYL)-	13633-25-5
3	22667	BENZALDEHYDE, 2-HYDROXY-3,6-DIMETHYL-4-(PHENYLMETHOXY)	34883-16-4
4	25594	BENZENE, 2-METHOXY-1-(2-NITROETHENYL)-3-(PHENYLMETHOXY	74810-83-6
5	9304	BENZENE, (2-METHYLPENTYL)-	39916-61-5

Fit	Elements	Bpk	Mwt	pur	MIX	rev
1	C12.H18.	91	162	259	650	298
2	C10.H13.BR.	91	212	257	865	277
3	C16.H16.03.	91	256	234	816	235
4	C16.H15.N.04.	91	285	225	843	258
5	C12.H18.	92	162	222	703	248

FIGURE 5: Library Search Report For Scan #141

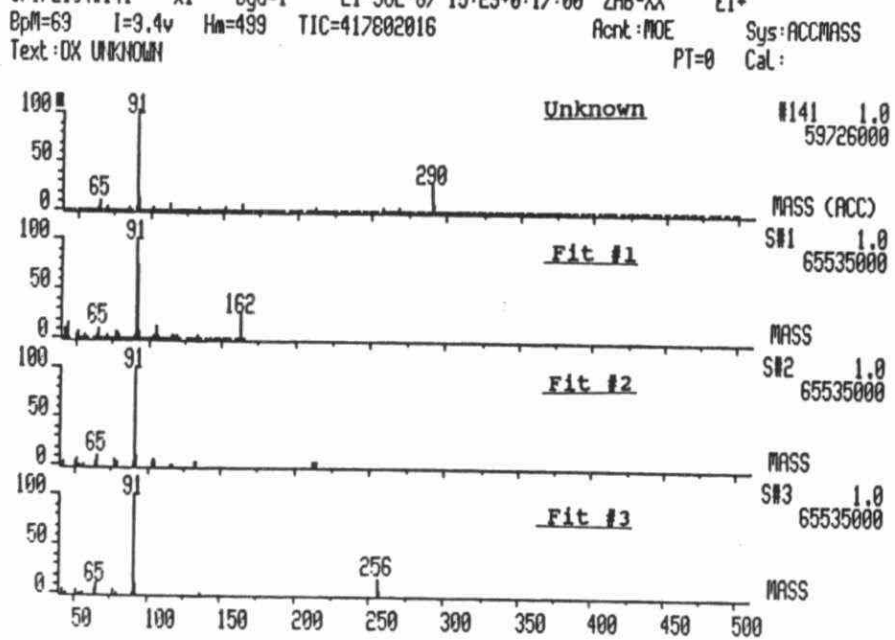

FIGURE 6: Comparison of Scan #141 with Library Spectra

W.D. #1554D

DEVELOPMENT OF THE GC/MED SYSTEM FOR HAZARDOUS WASTES

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1. INTRODUCTION

The application of the microwave induced plasma (MIP) as an excitation source for optical emission spectroscopy has been shown to be a useful element-selective detector for gas chromatography (1-5).

A unique microwave cavity described in the literature (6,7) generates plasma at atmospheric pressure utilizing either helium or argon as the plasma support gas.

Operation of the plasma at atmospheric pressure has facilitated the direct coupling of gas chromatographic effluent to the helium plasma to yield an element-selective gas chromatographic detector, the microwave emission detector, MED. Utilizing a multi-channel spectrometer and recorder, the need for high resolution chromatography is eliminated. While the system is sensitive down to parts per trillion levels, it does not have the capability for identifying specific compounds or isomers and this limits the method to screening purposes only.

The purpose of this communication is to report the results of studies on the GC/MED system as a fast screening analytical method for industrial effluents.

2. INSTRUMENTATION

The microwave cavity was constructed as reported in the literature (6,7) and it consisted of a cylindrical cavity made of copper with an internal diameter of 92.7 mm and a wall thickness of 10 mm. All internal surfaces were polished, silver plated and flashed with gold for protection. The microwave coupling loop was extended into the cavity with one end attached to the cavity wall and the other end to the Amphenol VG-58 connector.

The microwave generator (EMS, Microfron 200 Mark III) was coupled to the cavity via a coaxial line and double stub tuner (Model SF-3/N, Microlab/FXR). Impedance matching was accomplished by the double stub tuner placed directly before the cavity.

The light, emitted from the plasma, was focused onto the primary entrance slits by a single quartz condensor lens in an adjustable holder. The multi-channel spectrometer (MED 650) provides multi-element detection of the emitted light. The entrance slits, concave diffraction grating and all the exit slits are located on the Rowland circle. The light from the exit slit is then reflected by a plane mirror to the photomultiplier. The optical layout of the multi-channel spectrometer is shown in Figure 1.

The primary slit assembly consists of dual entrance slits, spaced 0.6 mm apart, each 0.1 mm in width and 3 mm in height. A chopper in front of the slits allows measurements to be taken on-line and off-line for automatic background correction.

The 500 mm spherical radius reflection grating has 1180 lines/mm and dimensions of 50 x 30 mm. Its 1st order

reciprocal dispersion is approximately 1.6 nm/mm. Diffraction order sorting filters, positioned in front of the photomultiplier tube holders, separate first diffraction order from the higher ones. The wavelengths selected to monitor halogen, phosphorus and carbon emissions are summarized in Table 1.

Two types of microwave discharge torches were investigated in our study. The capillary torch consists of 7 cm quartz tubing of 6 mm O.D. and 0.8 to 1.0 mm I.D. The Codding torch (8), a schematic diagram of which is shown in Figure 2, is made from two concentric quartz tubes arranged similar to a conventional ICP torch. The threaded quartz insert (4 mm O.D., 1.8 mm I.D.) generates a tangential gas flow which focuses the plasma and keeps it away from the wall and thus prevents the torch from being eroded at high power inputs. The thread is cut into the insert at a pitch of 10 and two threads of 0.5 mm wide and 0.6 mm deep are interspaced to yield a linear spacing between them of 1.27 mm.

The gas chromatograph (HP 5730A) was equipped with a 15 m J and W fused silica magabore column (DB-17, 1 um filter). The column was threaded through the heated transfer line and directly into the torch. The chromatograms were recorded on an RMS Instruments Limited, model GR33 multi-channel graphic recorder.

3. RESULTS AND DISCUSSION

The initial study was conducted with a capillary torch and a modified APL (model QA-137) high resolution monochromator. Being a high resolution instrument sensitivity was rather low (solid light collecting angle small) and the spectrometer built by Barringer Research (MED-650) was used for subsequent studies. The sensitivity and selectivity for the capillary

torch and MED-650 spectrometer for simple halogenated hydrocarbons was observed to be good as is seen in Table 2. Table 2 also summarizes the selectivity of the GC/MED system with respect to carbon. Figure 3 is a GC/MED multi-channel record of a number of organic compounds containing F, Cl, Br and P. Initial studies on the detection of chlorine have indicated a strong correlation between power and signal intensity. An example of this is displayed in Figure 4 for chlorine and carbon signals as breakdown products of chlorobenzene.

The capillary torch plasma was found to be stable only at low loading (about 0.025 uL) and low power. In order to prevent the plasma from extinguishing during the solvent elution, it was necessary to employ GC in the split mode (1:40 for a 1 uL injection sample). Thus, with pg/s sensitivity the actual concentration of the analyte in the sample for detection is in the nanogram per uL concentration range. In addition to problems associated with loading, the breakdown efficiency for more stable halogenated hydrocarbons such as PCB's was observed to be very low. Traces of the PCB breakdown were observed by increasing the power to 120 watts. However, at powers exceeding 85 watts the lifetime of the torch was considerably reduced and at 120 watts the torch lasted for only a few minutes.

The characteristics of the capillary torch requiring low sample loading and operating power present severe limitations with respect to the application of rapid screening of halogenated compounds. Hence, a microwave torch designed by Codding (8) was evaluated for high power operation. Figure 2 is a schematic diagram of the Codding torch. Gas introduced along the spiral of the inner tube acts as a coolant and plasma focusing gas. The GC column is terminated just before the end of the threaded inner tube and the torch is inserted into the

Beenakker cavity with the threaded end just before the cavity. In order to keep the plasma stable and focused, and to attain a quick plasma recovery after the solvent front, the coolant gas flow was maintained at about 500 cc/min with the auxiliary and carrier gas set to 50 to 80 cc/min. Both auxiliary and coolant gases were preheated in order to prevent analyte condensation. The schematic diagram of the experimental set-up is shown in Figure 5.

The Codding torch was observed to reduce the sensitivity of the MED by more than two orders of magnitude compared to the capillary torch for simple halogenated hydrocarbons. The reduction in sensitivity with the Codding torch is believed to be primarily due to a sample dilution, spatial distribution and residence time reduction of analyte in the plasma region. However, this reduction in sensitivity was largely offset by the capability of introducing a much larger loading into the Codding torch. The plasma could be sustained with sample loadings up to 10 uL with the GC in the splitless mode. Hence, no deterioration in detection limit was observed. No torch erosion was observed with power input of up to 180 watts.

At a power input of 180 to 190 watts, PCB's in the nanogram range could be detected. The GC/ECD and GC/MED chromatograms of Aroclor 1260 are shown in Figure 6.

The good correspondence between the GC/ECD and GC/MED chlorine peaks indicate that the PCB's are being broken down in the helium plasma. However, the decrease in the chlorine peaks and carbon peaks for higher chlorinated PCB's indicate that the breakdown is still inefficient even at 190 watt incident power.

Serious problems were also encountered with the lack of signal reproducibility. It was not uncommon to find a temporary loss of signal for the chlorine channel and then recovery of the

signal on the subsequent run. The lack of signal reproducibility with the Codding torch greatly hampered system optimization. It is believed that the lack of signal reproducibility is a result of the plasma's shifting position after solvent elution. The MED spectrometer operates with on-line/off-line background correction using a chopper disk with dual entrance slits. The signal from one slit represents the analytical line and the other slit is the background. Since background correction is performed in real time, errors could result if the plasma wanders in and out of the two slits. Currently, efforts to correct this problem are continuing.

4. CONCLUSIONS

The application of the GC/MED system to the rapid screening of hazardous wastes requires partial atomization prior to the microwave excitation of the target compound. Also, the system does not have the capability of identifying specific compounds and can only indicate the presence of absence of the target elements. For qualitative and quantitative identification a library of target compounds and their retention times and sensitivities would have to be established. The breakdown efficiency will be a function of both type of compound, the input microwave power, and degree of analyte breakdown prior to its excitation in the plasma, especially for very stable compounds like PCB's.

Experimentation to aid breakdown prior to excitation, different torch configurations to improve stability in spatial distribution and perhaps thicker cavities (2 cm thick cavity was used in the present study) to increase the residence time within the plasma region may improve both sensitivity and reproducibility. A modified method of bringing emission light onto the monochromator entrance slits, like fibre optics,

should also aid in better source focusing. Implementation of the solvent venting technique may also greatly improve the plasma stability. There are still many variables that need to be optimized before the system can be utilized as a detector for fast screening of industrial effluents.

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TABLE 1

Analytical and Background Correction Wavelengths in the MED 650

Element	Analytical Wavelength, (nm)	Background Wavelength, (nm)
C (2nd order)	193.09	194.07
P (2nd order)	213.62	214.61
Br (1st order)	470.48	471.48
Cl (1st order)	479.45	480.45
S (1st order)	545.39	546.39
F (1st order)	685.60	686.57

TABLE 2

Detection Limits and Selectivities
for Simultaneous Determination[1]
with Capillary Torch

Element	Compound	Detection Limit, pg/s[2]	Selectivity vs. C[3] Using n-decane for Carbon Signal
Br	$CHBr_3$	27	180
C	$C_{10}H_{22}$	4	1
Cl	C_6H_5Cl	12	200
F	$C_6H_4CH_3F$	11	366

(1) Plasma operating conditions 85 watts incident power, 0-2 watts reflected power, .50 mL/min He plasma support flow rate.

(2) Detection Limits (Signal/Noise=2/1) $= \frac{W_{analyte\ (pg)}}{W_{1/2}(s)} \cdot 2 \frac{h_{noise}}{h_{signal}} \frac{pg}{s}$
where $W_{1/2}$ line width at half maximum in seconds and W is the weight of analyte being monitored, e.g. Cl.

(3) Selectivity = (h/W)analyte/(h/W) carbon contribution on analyte from n-decane. It is assumed that half widths are the same. If different, correction as in (2) above must be incorporated.

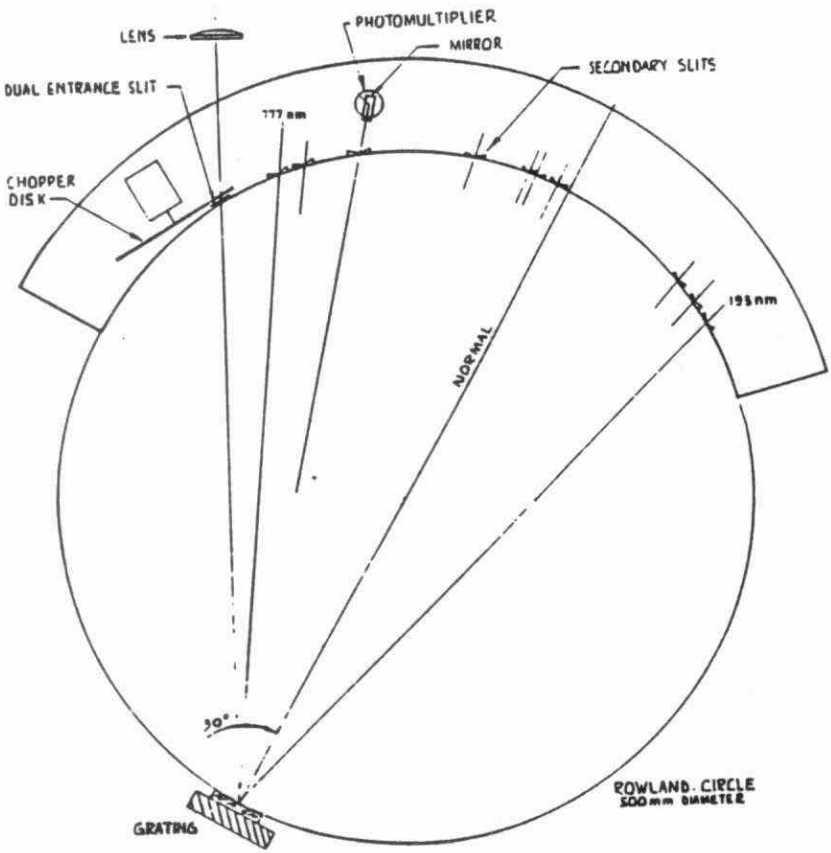

Figure 1 Optical Layout of Multichannel MED 650 Spectrometer

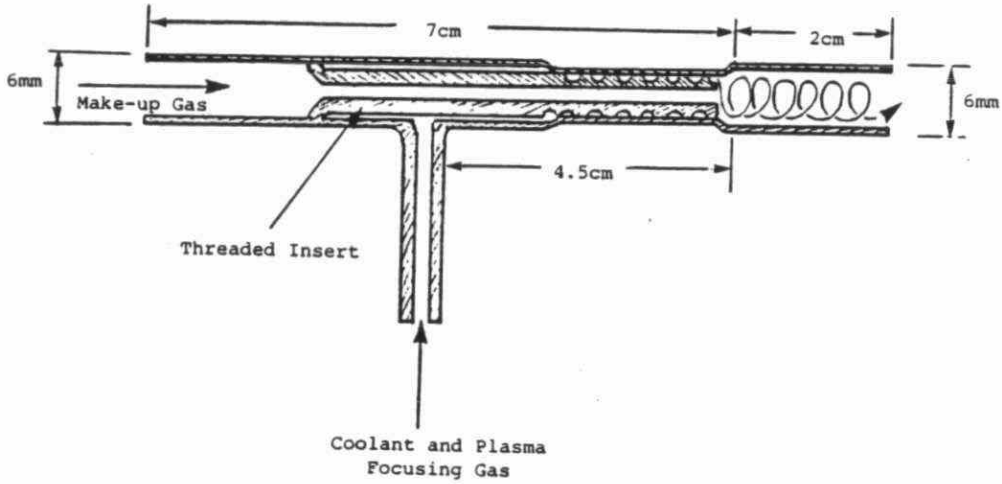

Figure 2 Schematic diagram of the Codding Plasma Discharge Torch

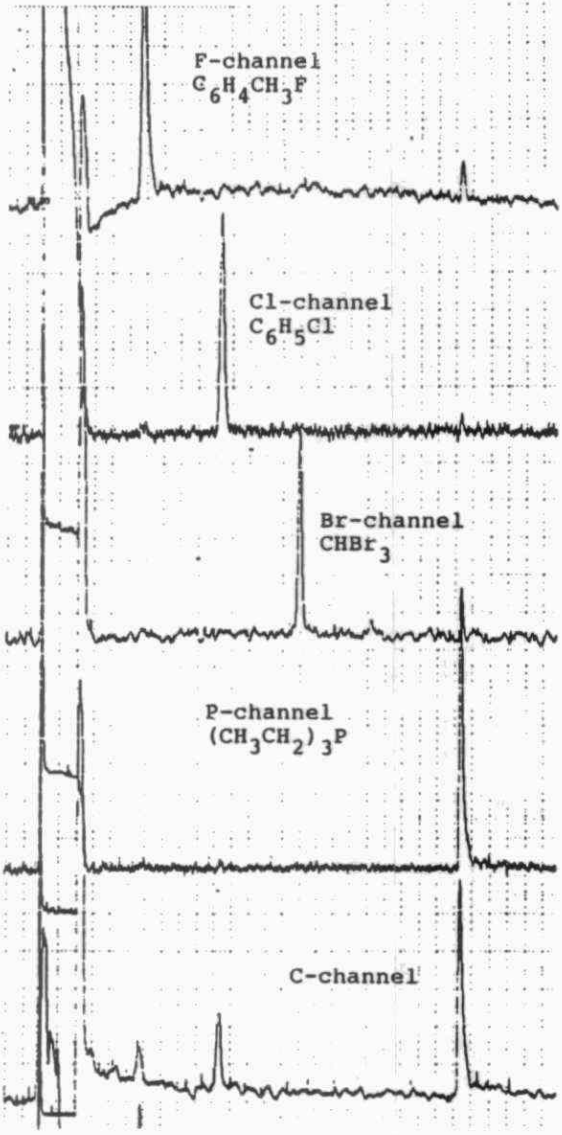

Figure 3 GC/MED Multichannel Record

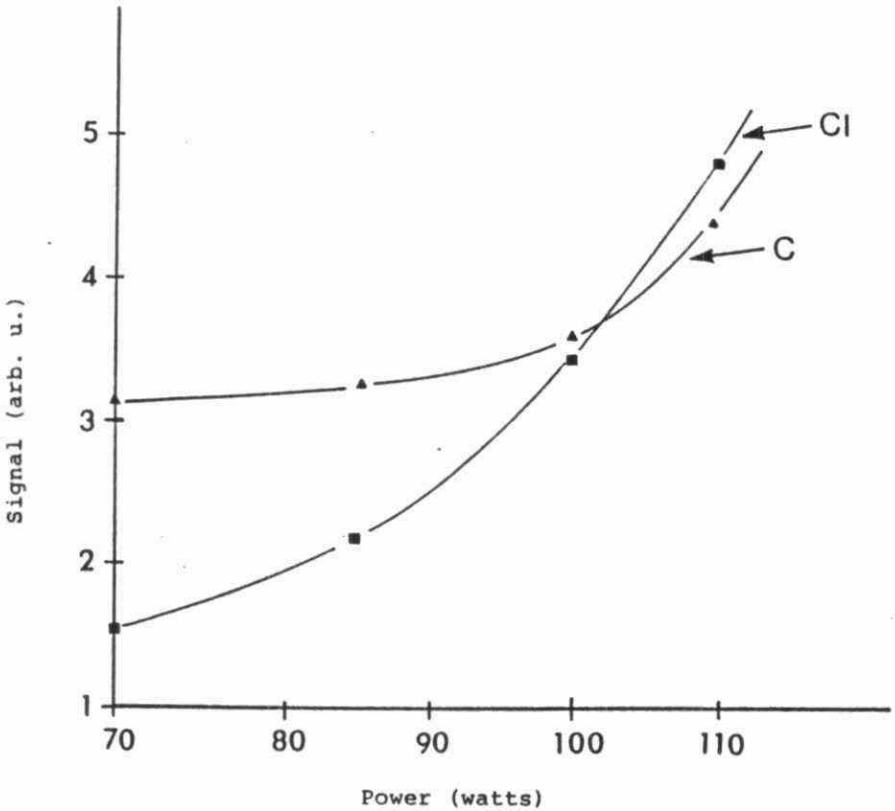

Figure 4 Chlorine and carbon signal from chlorobenzene as a function of incident power using capillary torch and MED 650 polychromator. GC conditions: Isothermal run at 100°C, with split ratio of 1:40, 1 ul injection volume of 60 ng/ul of Cl on 15 m megabore DB-17 column.

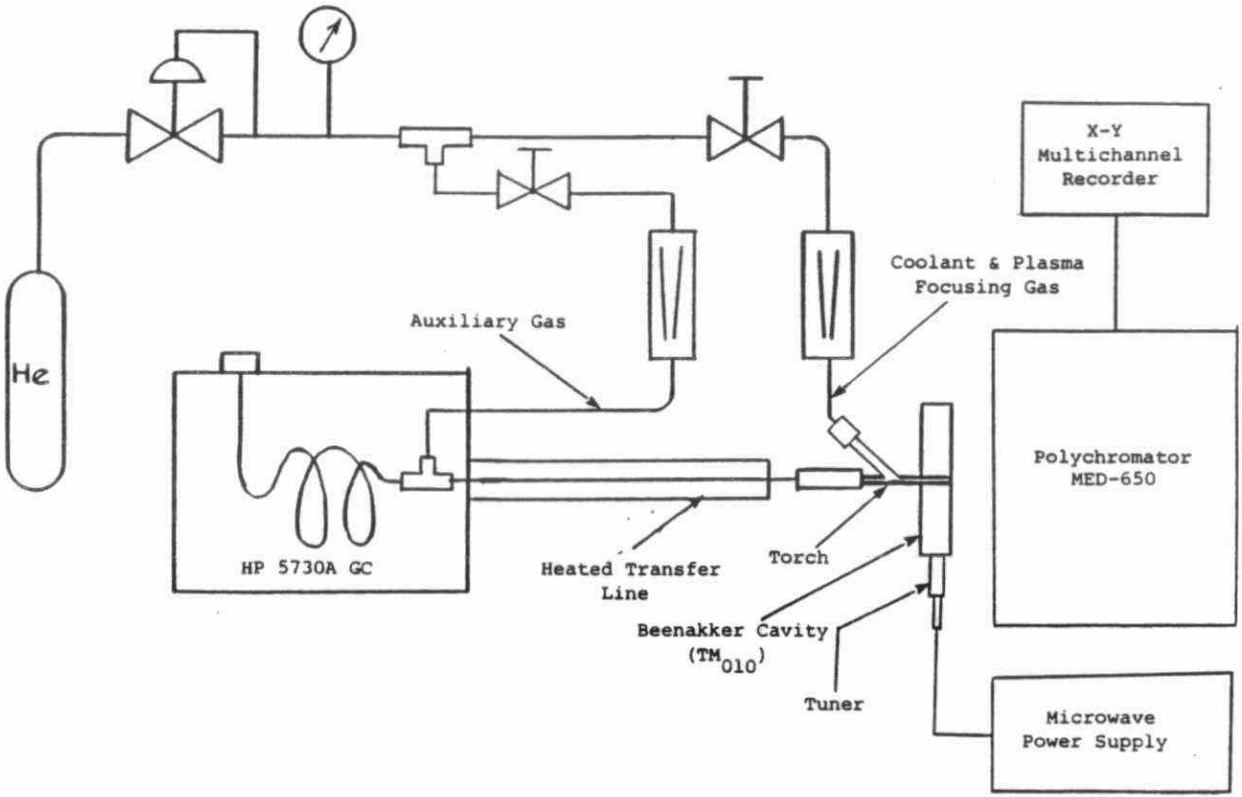

Figure 5 Schematic Diagram of the Experimental Set-up with Codding Torch

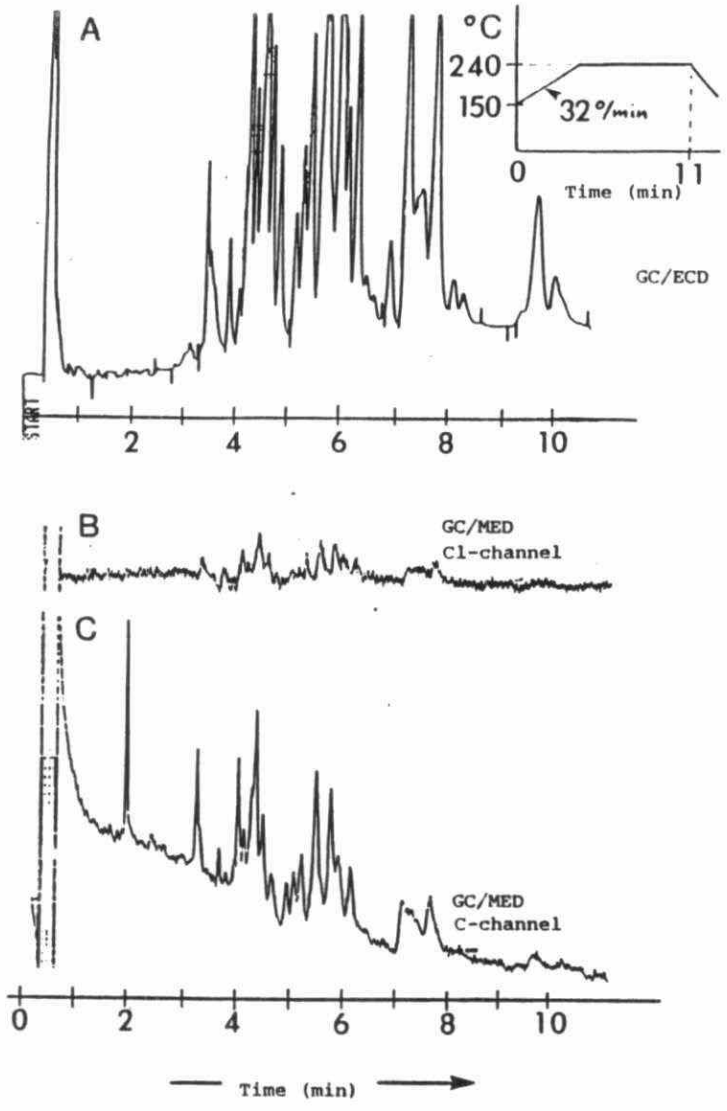

Figure 6 Chromatogram for Aroclor 1260
A - GC/ECD run with temperature programming as indicated;
B - GC/MIP run on chlorine channel;
C - Simultaneous GC/MIP run on carbon channel at incident microwave power of 190 watts and reflected power of 5 watts.

AN INTRINSIC CHEMICALLY SELECTIVE LIPID-BASED WAVEGUIDE FOR ORGANIC VAPOUR SENSING

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This work is directed towards the preparation and study of an intrinsic optical sensor which consists of a chemically selective fluorescent lipid biomembrane that acts as a light guide. Selective interactions of analytes with lipid, and receptors located within the lipid membranes, will produce perturbations of the membrane structure resulting in alterations of membrane fluorescence. The preliminary evaluation of the optical transmission properties of various membrane structures is reported, and correlated with lipid headgroup and acyl chain interactions, refractive index and film thickness. A surface stabilized multilayer which is able to operate as a wave guide is described.

INTRODUCTION

The majority of analytical measurements made today involve qualitative and quantitative evaluation of individual species in complex matrices, necessitating complete separation of sample components followed by appropriate analyses. This becomes increasingly difficult and expensive as sample complexity increases. Furthermore, this limits real time analysis, and direct analysis in certain major areas of interest, such as those related to long-term environmental monitoring.

All chemical sensors employ a basic union between a selective receiving site for ionic or molecular recognition, and a transducer which is capable of translating a perturbation of physical chemistry associated with the determinant-site reaction into a useable signal (1). Much emphasis has been placed on the development of biochemical selectivity by use of immobilized enzymes or antibodies. Major problems continue to exist in both the development of stable, highly sensitive and reversible chemically selective surfaces, and efficient marriage of the latter with various types of transducers (1).

Our basic research to date has concentrated on developing concurrent advances in each of the two major problem areas. The basic philosophy of operation has been to create an analogy of natural membrane-associated chemoreception, where the selective sensing membrane also acts as the transduction device (2). This work has clearly demonstrated that it is possible to prepare an artificial analogue of a natural lipid membrane which can contain a selective chemical receptor. A binding event between the receptor and a targeted analyte can alter membrane physical chemistry to

induce changes in electrostatic fields and packing/fluidity parameters, which directly control transmembrane ion current. The transduction event can be a translation of the membrane binding event directly into an altered ionic current through the chemically selective membrane, and can be used for analysis and control. The process does not rely on the development of equilibria, and formation of products from enzyme-substrate reactions or antibody-antigen interactions are unnecessary. This removes the chemical restrictions limiting the development of generic receptor structures and stabilized biomembranes. One of the major deficiencies of the electrochemical sensing strategy is the sensitivity to noise and external interference, and therefore significantly increased difficulty of applying this form of sensing in distributed or remote sensing configurations. Optical systems based on fibre optic technology can alleviate such problems since the analytical information can be transmitted over relatively large distances in an undistorted form. Furthermore, reference structures such as electrodes are not required, and optical systems can be self-calibrating (3). Numerous reports of such assemblies, known as extrinsic fibre optic sensors, have been previously reported (3-6). These generally suffer from the limited pathlength and primitive selective chemistry obtained by differentiating or attaching a selective wet-chemical solution cell to the end of an optical fibre. A much better strategy can be obtained by creating an intrinsic fibre optic sensor which is coated over the length of the fibre with selective reagent. An evanescent wave originating from the the optical transmission of the fibre and propagating into the selective chemical zone can stimulate chromophores or fluorophores (7). The result is alteration of the intensity, polarization and/or wave-length of the radiation propagated within the fibre as a function of the selective chemical changes occuring on the fibre surface. The selectivity of lipid biomembrane structures can be used in such an intrinsic fibre configuration by coating lipid membranes containing receptors and fluorophores on the surface of conventional optical light guides (8). However, the evanescent wave intensity is limited, and recapture of fluorescent excitation within a membrane by the fibre is limited, combining to reduce the sensitivity of such a device (9). This work has provided direction for an entirely new form of intrinsic fibre optic sensor.

The use of lipid membrane technology to create selective biomembranes and prepare physical and chemical models of natural chemoreceptive processes has demonstrated the advantages which can be achieved by combining the selective chemistry and the transduction process in one structure. Investigations studying optical transmission have demonstrated that carefully constructed multilayers of lipid can act as optical wave guides (10,11). This report describes recent advances in the structural manipulation of lipid membranes which will lead to development of an intrinsic optical sensor consisting of a selective biomembrane which acts as a light guide. This physical arrangement maximizes membrane selectivity parameters as well as optical emission and collection conditions, and integrates the chemistry and transducer into a single entity.

EXPERIMENTAL

Chemicals

Phosphatidylcholine from egg yolk (PC) (Avanti Biochemicals, Birmingham, AL, USA) and cholesterol (C) (Sigma Chemical Company, St. Louis, MO, USA) were used for phospholipid membrane formation. Stearic acid (Sigma) was employed to prepare fatty acid membranes. The fluorophores 4-hexadecylamino-7-nitrobenz-2-oxa-1,3-diazole (NBD hexadecylamine) and 12-(9-anthroyloxy) stearic acid (12-AS) (Molecular Probes, Inc., Eugene, OR, USA), were used as received without further purification. The membrane stimulants chloroform, n-hexane and n,n-dimethylaniline, and all other solvents were of analytical reagent grade. All water was obtained from a five-stage Milli-Q (Millipore Water System) cartridge filtering system and had a minimal specific resistivity limit of 18 megaohm-cm^{-1}.

Apparatus

A Lauda Model 1974 thin-film balance (Sybron-Brinkman Instruments, Toronto, Canada) was used in association with an in-house pneumatic film lift for deposition of lipid monolayers onto glass surfaces. Glass wafers were cut to dimensions of 0.5 × 3 cm from plain borosilicate microscope slides of thickness 0.1 cm (Fisher Scientific, Toronto, Canada).

Fluorescence measurements of monolayer-coated wafers were done with a Turner Model 111 fluorometer, (Palo Alto, CA, USA). The wafers were supported in a pyrex tube which could be rotated to provide reproducible excitation illumination angles. Gas phase experiments made use of a capped pyrex sample holder, which was connected to a thermally regulated headspace production vessel as shown in Fig. 1. Syringes provided for reproducible sampling and movement of small quantitative volumes of gas within the closed assembly.

Stearic acid multilayers which contained NBD hexadecylamine were prepared on commercial silicon wafers (Orientation N(100); 2" diam., Avrel Co., St. Charles, MO) for waveguiding studies. Characterization of multilayer thickness and structural regularity was done by ellipsometry (Auto EL, Rudolph Research, Flanders, NJ, USA) and microscopic observation. Fluorescence was obtained by irradiation of the wafer with light at 489 nm from an argon-ion laser (Coherent Innova 70). The optical arrangement for this experiment is shown in Fig. 2, and was designed to reduce the effects of scattered radiation, so that waveguiding properties could be reliably established. Fluorescence was analysed using a SPEX 1700II monochromator and an RCA 7625 photomultiplier tube operated at 2000 V.

Procedures

The phospholipid solutions consisted of 2.5 mg of PC and 2.5 mg of cholesterol (C) in 5.0 mL of hexane. These solutions were stored under nitrogen in darkness at -20°C when not being used. Fatty acid solution preparations consisted of 2 mg of stearic acid in 5.0 mL hexane, and were

stored as per the phospholipid solutions. The incorporation of the anthroloxy labelled fatty acid probe into the above solutions was achieved by first preparing a stock solution of fluorescent probe in a ratio of 1 $mg.mL^{-1}$ in ethanol. A fixed quantity of the ethanolic solution of approximately 10 to 100 ul volume was transferred to a separate vial and evaporated under nitrogen to dryness. The residual film was incorporated into the phospholipid or fatty acid solution by addition of a fixed volume of the hexane solution to the vial. Molecular ratios of fluorescent probe-to-lipid molecules in the ranges of 1:100 to 1:20 were investigated.

Lipid solutions were characterized by production of experimental pressure-area isotherms for lipid monolayers. Curves were constructed using the Lauda trough which usually contained a subphase of 900 mL of degassed water at a fixed temperature. Approximately 70 ul of the lipid solution was added dropwise over a period of 30 seconds to the aqueous surface (0.1MKCl) using a syringe. After a period of 15 minutes (to allow the hexane to evaporate) the monolayer was compressed at a speed of 0.9 $cm.min^{-1}$ until the collapse pressure was attained.

The glass wafers were cut by scoring with a diamond tipped scribe and then cleaving to the required size. The wafers were then sonicated in a 2% aqueous solution of sodium dodecylsulphate detergent for one hour, rinsed with distilled water, soaked in chromic acid for at least one hour, rinsed extensively with water and then stored in a dust-free environment. The PC/C monolayer on the trough was initially compressed to the desired surface pressure and allowed to equilibrate for 15 minutes. Monolayers were cast onto glass wafers at a surface pressure which was held constant during the entire casting procedure. A monolayer was transferred to a wafer by immersing the wafer by means of an automatic casting elevator at a speed of 0.85 $cm.min^{-1}$ through the air-water interface at an angle of 90°. At the end of the immersion cycle the wafer was allowed to soak in the subphase for 5 minutes before being withdrawn at 0.85 $cm.min^{-1}$.

The viability of the fluorescent membranes as gas phase sensors was demonstrated by providing variable atmospheres containing membrane perturbants and fluorescence quenching agents such as n,n-dimethylaniline for the anthroyloxy probe.

Stearic acid multilayers were prepared on the surface of cleaned silicon wafers. Wafers were cleaned in a solution of ammonia:hydrogen peroxide:distilled water (1:1:5) at 80°C for 5 minutes followed by a solution of HCl (conc.):hydrogen peroxide:distilled water (1:1:6) for 5 minutes, then rinsed several times with distilled water. Monolayers of stearic acid or phospholipid were prepared on the Lauda trough as previously described. The silicon wafers were then immersed and withdrawn from the subphase through the monolayer by the casting elevator at a velocity of 1 $cm.sec^{-1}$. The subphase was a 0.1M KCl solution in all cases. The film casting was continued in a cyclic manner (to prepare waveguides) during which the wafer was immersed and then withdrawn from the trough subphase through the monolayer, (maintained at a pressure of 30 $mN.m^{-1}$). The surface of the wafer was dried in air before each subsequent casting cycle was done. Periodically, the relative thickness

of the deposited film on the dry wafer was measured by ellipsometry. Mixed lipid multilayers in which the uppermost layers contained NBD hexadecylamine or PC, were prepared by replacing the previous monolayer with the new chemical mixture.

RESULTS AND DISCUSSION

Membrane Perturbation in the Gas Phase

The initial concentrations of the various gases used for experimentation were > 20 ppt (parts per thousand) chloroform, 200 ppt hexane and 2 ppt DMA. Response was noted as the gas sample was drawn from the sampling flask through the wafer holder and into the receiving flask. The membranes used were PC/C monolayers containing the 12-AS probe deposited onto glass wafers.

A large fluorescence increase was obtained from PC/C monolayers containing 12-AS upon exposure to air, chloroform and hexane. This response was almost always transient, returning to the baseline value within one minute. This was observed for chloroform concentrations as low as 25 ppt. The transient behaviour is likely due to a process such as a phase transition, where the presence of interfacial regions between phases causes an increase in fluorescence. This transition would be expected to occur from a more dense to a less dense phase on incorporation of the organic molecules, altering the overall structure of the membrane. Similar transient phenomena have been observed for lipid membranes in electrochemical (12) and piezoelectric (13) experiments, where transitory alterations in dipolar potential and microviscosity occur on exposure of the membrane to non-selective interaction with probes.

The reagent N,N-dimethylaniline (DMA) is a known quencher of the anthroyloxy probes (14) and caused a significant decrease in the fluorescence signal. Again, the signal was transient, and a rapid significant decrease in fluorescence was observed for one minute, followed by a signal increase to some steady state, usually less than the initial fluorescence. The decrease in fluorescence may be related to the concentration of the quencher [Q] by the Stern-Volmer equation (15):

$$\frac{F_0}{F} = 1 + K[Q] \qquad (3)$$

Where K is the quenching constant, and F_0 and F are fluorescence intensities of the fluorophore in the absence and the presence of the quencher respectively. The Stern-Volmer equation is only valid for purely dynamic or collisional quenching occuring in the absence of any significant inner filter effects. The inner filter effect arises when the quencher absorbs either at the wavelength of excitation or emission of the fluorophore.

A Stern-Volmer test for DMA is shown in Table 1 and indicates that a linear relationship with a correlation factor of 0.89 was obtained over a significant concentration range.

The fact that a linear relationship was obtained rules out any significant inner filter effect. The Stern-Volmer data do not indicate whether dynamic or static quenching is occuring.

These chemical interactions with the lipid membrane and the fluorescent probe demonstrate that analytical signals can be generated by events which perturb the membrane structure. Chemical selectivity for distinct classes of compounds can be achieved by methods such as fluorescence quenching. Further optical information in the form of wavelength and fluorescent lifetime measurements can be used to identify and quantify non-selective interferences. However, the potential for "ideal" chemical selectivity can only be achieved by the use of formal selective binding interactions invoking receptors.

For receptors to be active as fluorescence modulators, they must be either conjugated to an appropriate fluorophore or they must significantly affect a fluorophore embedded in the lipid membrane matrix. Significant results delineating covalent fluorophore conjugation have already appeared in the literature (16-19) and demonstrate the feasibility of this approach. A second method of receptor control of fluorescence signal involves the manipulation of membrane electrostatic fields and fluidity/packing by short range interactions of the receptor or receptor-stimulant complex with membrane lipids. Consequently lipid membrane embedded fluorophores which are sensitive to internal membrane potentials and order will be affected by receptor-stimulant complexation. Such interactions have been observed by techniques such as epifluorescence (20) and provide substantial encouragement for the development of sensitive sensing stategies.

Lipid Multilayer Waveguides

Sweep barrier movement of the Lauda trough, direct reflection ellipsometry, and water contact angle measurements were used to monitor monolayer deposition. Due to the nature of the direct reflection ellipsometry experiment, a highly reflective surface was necessary, and was obtained from the surface of a silicon wafer. The bare wafer was characterized using the ellipsometer and measurements were made of the surface after each subsequent casting cycle to establish a calibration curve for the relative film thickness in contrast to the number of monolayers which were deposited. It was established that this was a linear relationship (Fig. 3) to at least 215 layers with a correlation coefficient of 0.999.

At low compression pressures the stearic acid was not aligned on the surface of the trough, though polar interactions with the subphase were expected with the carboxylic acid headgroup. During compression, the hydrophobic acyl chains became restricted with respect to three dimensional motion and the molecule as a whole became restricted with respect to lateral motion, until the head groups achieved maximum packing density and the acyl chains attained maximum vertical extension. Further lateral compression caused localized formation of undesirable multilayer regions on the surface of the trough.

The rapid monolayer casting process insured that the film could be held at a lower pressure than was necessary at the slower speed ($0.1 cm.sec^{-1}$). Casting at such lower pressures avoided the structural variability of monolayers. The first layer deposited was aligned by polar bond formation between the hydrophilic sites of the molecule and the silicon oxide surface, and occured during removal from the subphase. The dense packing of the transferred monolayer caused the new surface to be hydrophobic as demonstrated by contact angle measurements. Subsequently, casting caused deposition to occur during both immersion and withdrawal through the monolayer. These layers deposit due to Van der Waals interactions between acyl chains, and polar interactions between the carboxylic acid headgroups and retained water of hydration. Barrier movement was constant for each monolayer deposition and allowed a transfer ratio of approximately 0.98 ± 0.03 to be established, (estimated due to the irregular shape of the wafer). Contact angle measurements indicated that the orientation of the surface layer of stearic acid molecules always assumed a configuration in which the acyl chains weredirected away from the silicon wafer surface.

The long term stability of the multilayers when stored in air or aqueous solution was excellent, and no apparent thickness/refractive index alterations were observed ellipsometrically over periods of weeks.

The structural characteristics of the multilayers seemed to alter at a thickness of approximately 10 monolayers, as detected by an alteration of the visual appearance of the meniscus formed at the boundary between the wafer surface and the trough solution surface (initially only positive; subsequently negative on immersion and positive on withdrawal from subphase). These results may indicate a change of angular orientation of the multilayers with respect to the supporting substrate. The ellipsometric evidence indicates that the effect was not of significance with respect to the refractive index or thickness of the film. The drying of the multilayer between casting cycles provided an opportunity for water to drain down the face of the wafer and pool near the botton before evaporation. This caused some microscopic structural imperfections in certain areas of the wafer, particularly near the centre and lower edge.

Low magnification optical microscopy using optical reflectance indicated the presence of small bright centres (diam. 0.01 mm) which were distributed quite densely throughout the multilayers. These were likely caused by the inclusion of small crystals of KCl (appeared when water had been evaporated). The removal of these crystals was accomplished by rinsing the wafer with distilled water before drying between casting cycles. The relative physical perfection of the multilayers has been improved by rapidly drying the wafers between casting cycles with a jet of warm air, thereby avoiding problems of solution flow across the surface.

The sequential deposition of 200 monolayers of stearic acid provided the base for the preparation of a fluorescent waveguide. Stearic acid has a chain length of approximately 4nm, which would indicate an approximate thickness of 800nm for the multilayer system, if the system is assumed to have no angular dependence and no interdigitation. This thickness would allow for

visible light from the argon laser to be guided by total internal reflection through the lipid matrix in a monomode capacity. To determine if waveguiding was occuring, 20 layers of stearic acid containing the fluorescent probe NBD hexadecylamine were deposited onto the 200 stearic acid layers. The optical arrangement for this experiment is shown in Fig. 2. The angle of illumination of the wafer was varied by moving the optical fibre to monitor the change in fluorescence intensity of the probe, which could only be related to the event of waveguiding. The results of this angular dependence of fluorescence are summarized in Fig. 4, and indicated an angular threshold of between 42° and 45° for initiation of the waveguiding effect. The critical angle was calculated to be 42° on the basis of a refractive index of 1.5 for stearic acid, and 1.0 for air. The decrease in signal at 70° is consistent with a decrease in the quantity of light entering the waveguide due to increased scattering.

The production of chemically selective multilayers formed by depositing a receptor-doped phospholipid monolayer matrix onto an underlying stearic acid waveguide has been initiated. The hydrophobic nature of the surface of the multilayer assembly provides an ideal surface for deposition of a phospholipid monolayer in a biologically significant orientation. Transfer of phospholipid monolayers to such surfaces by Langmuir-Blodgett casting techniques have been done, as shown in Fig. 5. Transfer of a phospholipid monolayer containing acetylcholine receptor onto solid substrates has also been accomplished. The lipid membrane system would transmit excitation radiation by total internal reflection, and would collect and transmit fluorescence similarly. Fluorescence should be readily measured since the emitted radiation is wavelength shifted in comparison to source radiation, and furthermore could be measured relative to a dark background if a pulsed source and phase sensitive detector are employed. The location of the fluorophore-receptor complex in the lipid membrane insures efficient capture of fluorescent radiation. A laser source is preferrable due to its very high intensity, which could potentially overcome fluorophore quantum yield deficiencies. A neutral density filter system would be used to optimize the fluorescence signal and control protein denaturation which could occur at high optical excitation intensities.

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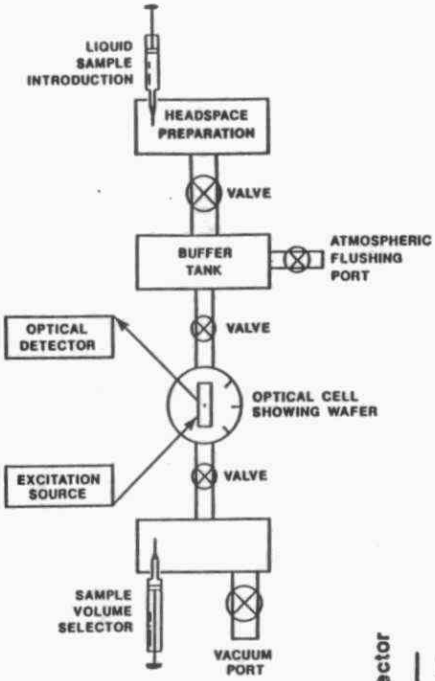

Fig. 1. Gas phase sampling apparatus showing location of production of headspace of defined partial pressure and sample wafer housing illustrating excitation beam alignment.

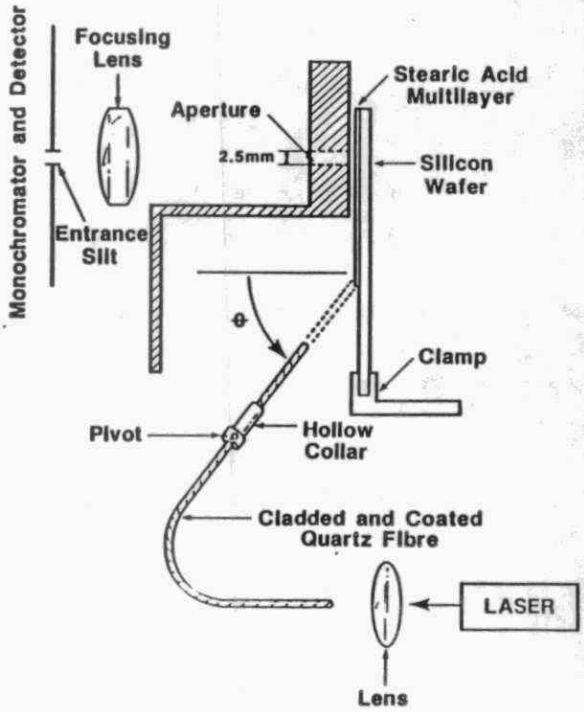

Fig. 2. Apparatus for testing the critical angle for wave-guiding by a lipid multilayer supported on a silicon wafer. The optical fibre can be manipulated to achieve various angles of illumination (θ).

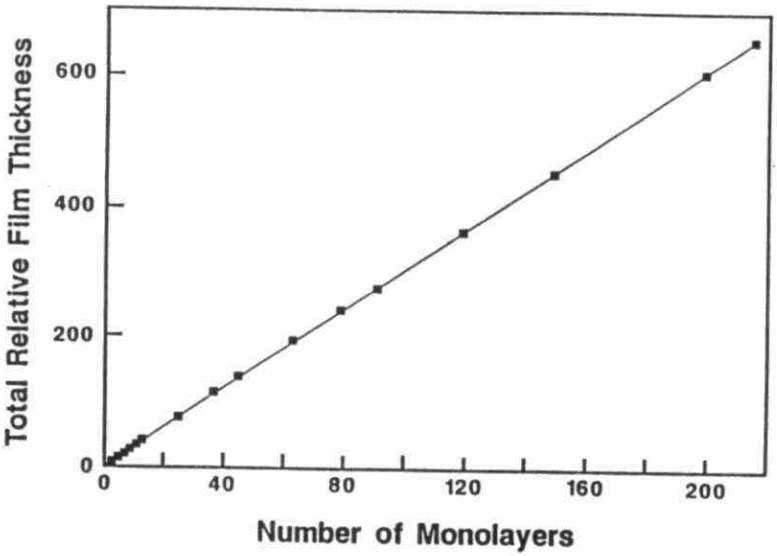

Fig. 3. Correlation of the relative lipid multilayer film thickness (as established by ellipsometry) to the number of monolayers deposited on a silicon wafer.

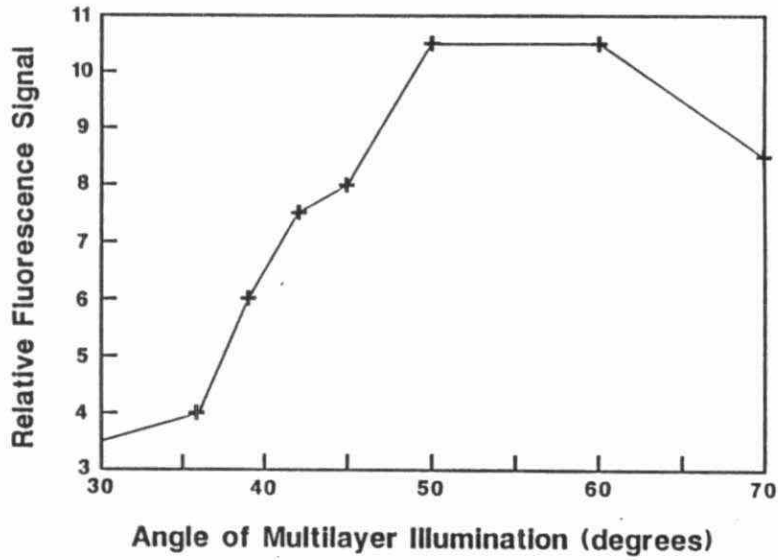

Fig. 4. The angular dependence of waveguiding indicated by the onset of fluorescence within a stearic acid multilayer; assembled from 200 monolayers of stearic acid, with an external coating of 20 monolayers of stearic acid containing NBD hexadecylamine.

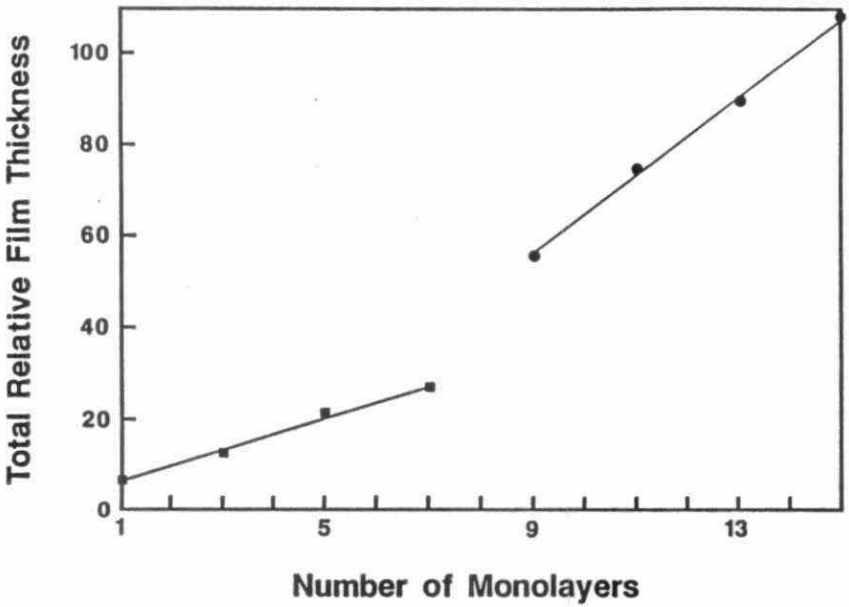

Fig. 5. Ellipsometric characterization of phospholipid monolayers deposited on a base consisting of a stearic acid multilayer. The chemical composition of this assembly represents that proposed for selective sensor development using molecular receptors.

■: Stearic Acid

●: PC/C

Table 1: Stern-Volmer data for the fluorescence quenching agent n,n-dimethylaniline acting on *12-anthroyloxy stearic acid

Dimethylaniline Concentration (ppt)	F_o/F
0.1 ± 0.1	1.002 ± 0.002
0.5	1.009
1.0	1.012
1.4	1.019
2.0	1.036

*The fluorescent matrix was a monolayer of phospholipid containing 12-AS (22:1 molecular ratio of lipid-to-probe) deposited at 35 $nM.m^{-1}$ onto a borosilicate glass wafer.

Development of ACexpert. 1. Design of an Expert System for Automated Metal Analysis by Atomic Absorption Spectroscopy.

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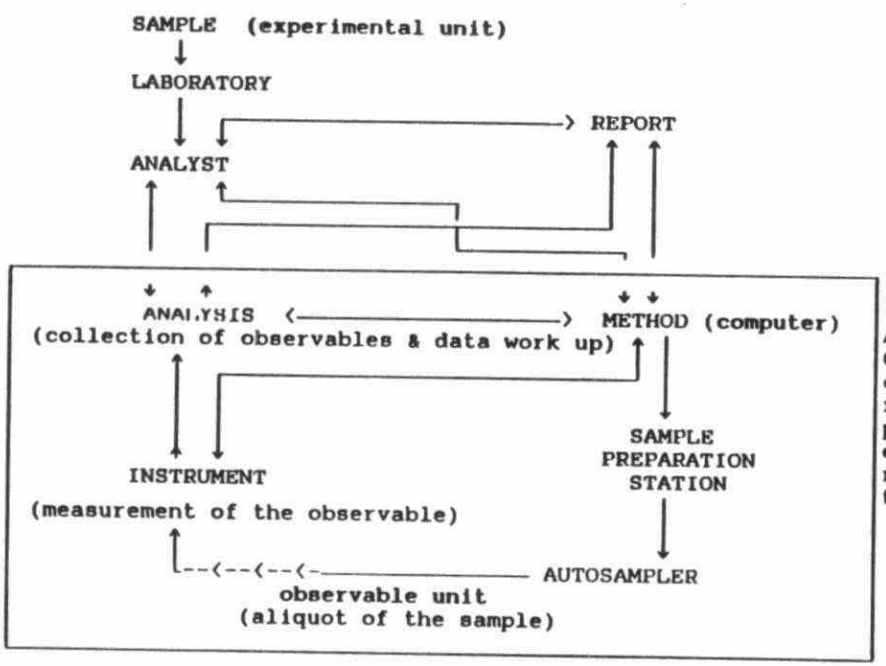
Abstract

The objectives of this research study are to develop an expert system that can be used to control all aspects of metal ion analysis carried out by the technique of AAS. The system will provide (i) real-time control of solution preparation, sample dispensing and sequence control from an autosampler, (ii) control of the instrumental settings and data acquisition, and (iii) an estimation of the quality of the data obtained by the instrument. On the chart drawn below, arrows represent the flow of the sample through the laboratory, the flow of analytical information, or the flow of process control information. ACexpert is being designed at present to provide complete control of all features contained within the box.



Introduction

Automation of the analytical laboratory (2), analytical instrumentation (3,4,5,6), and process analytical chemistry (7) is a component in the advancement of productivity. For laboratories, which must manage large amounts of data, maintain complex quality assurance programs, and control instruments in real-time (8) the integration of expert systems into the automation processes will become increasingly important (9). In general at present, expert systems are used in separate interactive applications involving consultations, such as diagnosis or prescription, prediction, monitoring and control, planning, instruction, and interpretation. However, considerable pressure is being applied toward integrating expert systems with existing information technologies, such as numeric computation, databases, and simulation (14).

Design features for ACexpert

The main objective in the development of **ACexpert**, is to provide assistence to the user and manager of a quality assurance program for metal analysis. More specifically, the purpose of this expert system is to provide consistent, high quality, well-documented determinations of metal concentration in a variety of samples using atomic absorption spectroscopy (AAS) by automating both the analytical procedures and the QA/QC program. In addition, this system will be used to monitor and model quality control procedures used for instrumental data analysis, with specific applicaiton to the analysis of metal ions by AAS.

In detail, the **ACexpert** system will provide (i) real-time control of solution preparation, sample dispensing and sequence control from an autosampler, (ii) control of the instrumental settings and data acquisition, and (iii) an estimation of the quality of the data obtained by the instrument. Keeping in mind the additional requirement that the system must be used by experts and novices alike, these requirements impose design constraints on the system. Specifically, the computer system must be able to handle many tasks simultaneously in order to monitor and control the instruments. The system must also be able to solve problems based on a knowledge base of facts and solution strategies, and the system must have a human-machine interface that is simple to use, but at the same time explains thoroughly the process, which is being carried out, and status of the system. From the point at which the sample enters the laboratory to the point that the report is written for the client, **ACexpert** will assist in the completion of the analytical procedures.

ACexpert is not a single expert system, but a collection of systems which will include several types of experts that will be integrated to produce an automated chemistry workstation. At each step of the process the computer system will monitor the progress using an expert system that will be built from a series of modules, each of which will be limited to a relatively narrow domain of

expertise. However, together, these modules will encompass the many varied aspects of decision-making that would normally be carried out by the human expert: from methods selection, to real-time process control and then to reporting and documentation.

Implementation of the **ACexpert** concept as outlined above requires a system design process that includes thorough prototyping and validation before a production system is completed. The development of **ACexpert** will proceed through several stages of prototyping. As starting points, we selected for development the specific operations of instrumental control of solution handling and the expert system knowledge bases. The instrumental control program and the user interface development has proceeded along a traditional route using the Microsoft Windows (MS Windows) multitasking and graphical interface environment and writing the procedures in the C computer language. The development of the expert system knowledge base has proceeded by using expert system shells (1,11) that contain specific strategies for knowledge representation, inference, and user interface. Two expert shells are being used: KDS3 (KDS Corporation) and Cxpert (Software Plus Ltd). KDS3, which requires only that the user specify the expert knowledge in terms of examples of conditions, conclusions, and the relationship between them, will be used to construct a rule base and an expert system using its built-in inference and search strategies, and user interface. Cxpert, on the otherhand, will be used with the C procedures and user interface of the instrumental monitoring and control modules, and then linked to the rules and knowledge bases that are developed with the KDS3 system.

Design of the Expert Systems

Unlike traditional computer systems that attempt to solve problems with well defined procedures, expert systems use deductive reasoning to solve problems. Expert systems simulate the problem solving process of human experts and consist of computer hardware and software that can help advise, diagnose, analyze, consult, and categorize. These programs are tools for problems that normally require the use of a human expert. Three of the more important types of expert systems, which will be used in the **ACexpert** system, are the consultation, interpretation, and monitoring and control expert systems. In our design, **ACexpert** will consist of three compenent expert systems, **ACmethods**, **ACcontrol**, and **ACanalysis**. **ACmethods**, will encompass fault recognition and identification, and methods selection, and will use the consultation and interpretation type of expert systems. The monitoring and control, and interpretation type of expert systems will be combined into the analytical process control expert, **ACcontrol**. The quality control and quality assurance expert, **ACanalysis**, will be based upon a interpretation type expert system.

Each expert system module will be made up of four essential components: (i) a knowledge base of facts and rules, (ii) a problem-solving inference engine, (iii) a knowledge aquisition

module, and (iv) an explanatory interface (10). In turn, each of these modules with be tailored to the task that they are to perform. Clearly the knowledge acquisition module and interface module will be very different for the monitoring and control experts and the methods selection or data interpretation experts. The knowledge base contains symbolic knowledge, such as a description of the objects, their attributes and corresponding data values. As an example, the ACcontrol knowledge base will contain a description of the instrument components, ranges of valid settings, and lists of error codes. In addition, the knowledge base will contain rules that consist of judgemental knowledge such as the relationship between the gas flows of an AAS and the stability of the flame and sensitivity of the spectrometer. The inference engine is the component of the expert system that allows rules and logic to be applied to facts in the knowledge base. The rules may be analyzed by two different strategies, forward-chaining and backward-chaining that may be constrained by selecting a search technique. Forward-chaining solves problems by asserting new facts or conditions, and examining the consequences or conclusions. The backward-chaining inference strategy attempts to solve problems by hypothesizing a conclusion and then examining the truth of the conditions that are associated with the conclusion.

ACmethods: A Consultation Expert for Analytical Methods Selection

The KDS3 expert system shell is being used as a development tool to produce the separate expert modules, such as **ACmethods**. Specifically, the KDS3 shell is being used to construct and test the rules and facts that will become part of the system knowledge base. However, once completed, each KDS3 module could be used as a standalone expert system. A prototype version of the **ACmethods** expert has been developed to assist the user in the selection of the appropriate method for the analysis of metals based on the procedures included in the Environment Canada NAQUADAT dictionary (15). The selection of the appropriate metal analysis methodolgy as specified by a particular regulatory agency is the first step in the analytical quality assurance program. **ACmethods** uses a forward-chaining strategy and the following information as conditions: type of analyte, technique, sample type, regulatory agency, and if necessary, details of the methods to discriminate between similar methods.

It should be noted that this expert system was developed in much the same way that it is used. The developer is prompted with questions about analysis conditions, until a conclusion, in the form of an analytical method, is reached. If the KDS system selects an analytical method that is not the analytical method that developer had in mind when the questioning began, the developer is prompted for a new analytical method by the KDS system, and a condition that would distinguish the KDS analytical method from the developer's analytical method. The developer continues this process until all the analytical methods are considered and distinquished from each other.

An example of the use of an analytical methods selection expert, which was developed with the KDS3 system, is provided below. From the main menu, Aluminum is selected as the analyte of interest. The KDS shell then prompts the user with a series of questions that can be answered with Y (yes), N (no), or ? (don't know or don't care).

1. Analysis of dissolved metal in an aqueous sample? Answer: N

2. Analysis of mineral acid extractable metals? Answer: N

3. Use the flame AAS technique? Answer: Y

4. Analysis of metal in suspended material of sample? Answer: N

5. Analysis of metal in sediments? Answer: Y

6. Metal extracted with solvent to eliminate interferences? Answer: N

7. Required by the Water Quality Branch, Ottawa? Answer: Y

To give:

Title: Actinium to Carbon	Sample type: total sediments Sample prep: Open digestion with HNO3, HClO4, and HF. Analysis: Flame atomic absorption by direct aspiration, measured spectrophotometrically at 309.3 nm and compared with identically prepared standards. An acetylene-nitrous oxide reducing flame is used. Detection limit: 1 mg/kg Required by: WQB Ottawa. **** Approved WQB Method ****
THIS IS MY BEST ANSWER. 13053 Al, Flame AAS, total sed., required by A-WQB-O.	

kds

If question 7 was answered ambiguously with a ? (don't know or don't care), then two answers or methods would be presented and the system would warn the user that insufficient information was provided to resolve the differences.

Title: Actinium to Carbon	You may press the BACKSPACE key if you wish to go back and see if you can answer any or all of the unresolved Conditions which you previously answered with a "?". Conditions to which you already gave a Y or N answer will not be asked again except the last one.
Possible conclusion(s): 1 13053 Al, Flame AAS, total sed., required by A-WQB. 2 13053 Al, Flame AAS, total sed., required by A-WQB-O. **I have been unable to resolve 1 condition(s) either directly from the answers or through inference. As a result please regard the above as conjecture, not advice.**	

kds

When the expert system was being developed, if the developer had been confronted with ambiguous conclusions, then the KDS development system would have prompted the developer to select a condition from the existing set of conditions or input a new condition that would

have resolved the ambiguity.

ACcontrol: The Monitoring and Control Expert

ACcontrol is a system that must function in real-time using procedures for instrumental control that are described in the next section. This expert system must be structured so that it has access to the appropriate information at the right time from the sensors of the instrument, and structured so that decisions are made quickly. Specifically, the decision-making and supporting calculations must occur within the cycle time for the sampling. Combining the monitoring, control, and interpretation tasks of the expert systems requires that individual tasks be performed at the same time, either on separate computers, which can communicate with each other, or within a multi-tasking environment on a single computer. In either case, a great deal of importance is put on computational and reasoning efficiency. Generally, the system must have complete information to allow the interpretation expert to return a definite answer to the controlling expert. The occurrence of incomplete information, long reasoning chains, unreliable data, and contradictory input information are difficult for this type of expert to handle. Any of these situations should trigger the fault-finding expert and send an alarm to the operator.

Instrumental Control

Before the monitoring and control expert system can be implemented, a traditional procedure based user interface, a data base manager, and instrument controlling programs must be produced. The hardware of the **ACexpert** system consists of a Gilson 222 Autosampler and 401 Diluter, and a Varian 875 AAS that all are controlled by an AT-compatible microcomputer. The autosampler and diluter act in concert to provide solution preparation and sample injection into the AA spectrometer. The user interface makes extensive use of graphical displays to control the instrument setup, sample setup, and metal determination procedures in an interactive environment based on MS Windows (Microsoft Corporation). This graphical user interface is similar to the type depicted as a "Human Processor Interface for ICP-AES" by Karanassios and Horlick (12) or as presented for the automation of a chromatography workstation (13).

User and Instrument Interface Development

The user and instrument interface programs have been created as an MS Windows set of applications that can be run at the same time within the MS Windows multitasking environment. Each MS Windows application has access to all the features of MS Windows such as pull-down menus, and mouse driven windows and icons. All of these features are necessary to provide a simple to use, robust system for instrumental control. It is worth mentioning here that Windows is in fact not a true multitasking system. In other words, there is no scheduling program that ensures a particular application won't use

all the CPU time. Rather, it is up to the application to yield control to the other applications periodically.

Instrument control, sample preparation and injection into the AAS are performed by two MS Windows applications. The first, called **AAS**, is responsible for providing a computer interface to the Varian 875 AAS. This application provides the user with a "Control Panel" (see fig 1) that will allow control of the AAS operating parameters. Using a mouse, the user simply clicks the appropriate control button on the screen and the application (through a RS-232 hookup) sends the AAS the command. The user is able to save the instrument setup in a disk file which may retrieved for future use or printed on a printer.

The second application, called **Sampler**, is the heart of the instrumental control application. This application sets up three window procedures to handle (i) control of the Model 222 Autosampler and the Model 401 Diluter as slave devices, (ii) the sequencing of the batch, and (iii) the data values received from the AAS following every measurement. Control of the Gilson devices is carried out as follows. Each device is assigned a unit number on the GSIOC, (similiar to IEEE specifications) and the computer acts as the master device. Each device is programmed in it's primitive mode. This mode allows <u>all</u> of the features of the device to be used. Sequence control is performed by providing the user with a picture of the rack they will use, and by using the mouse. The user designates which tube contains the standards, samples, blanks or controls. Also, containers for larger amounts of blank or reslope solution may be put on the sampler tray, these too are displayed on the computer screen in the way they appear on the sampler tray (see fig 2 and 3). The data-feedback-window procedure (see fig 4) reads the data that appears on the RS-232 line from the AAS and dispatches the data to two other procedures: (i) data output and (ii) the quality control procedure. The data output procedure writes the data to the screen, the disk file or the printer. The quality control procedure will calculate the sample's relative standard deviation and decide if the sample should be reanalyzed.

Along with these two applications, a utility program has been developed called Rackedit. The program allows the user to enter data about new Gilson racks they have obtaind or to create custom racks (see fig 5). The information about the racks is stored in a file and is used by the Sampler application to locate the sample tubes.

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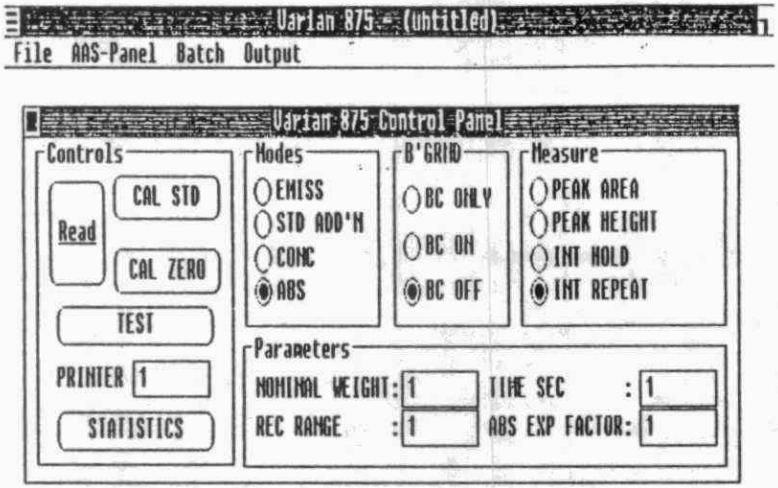
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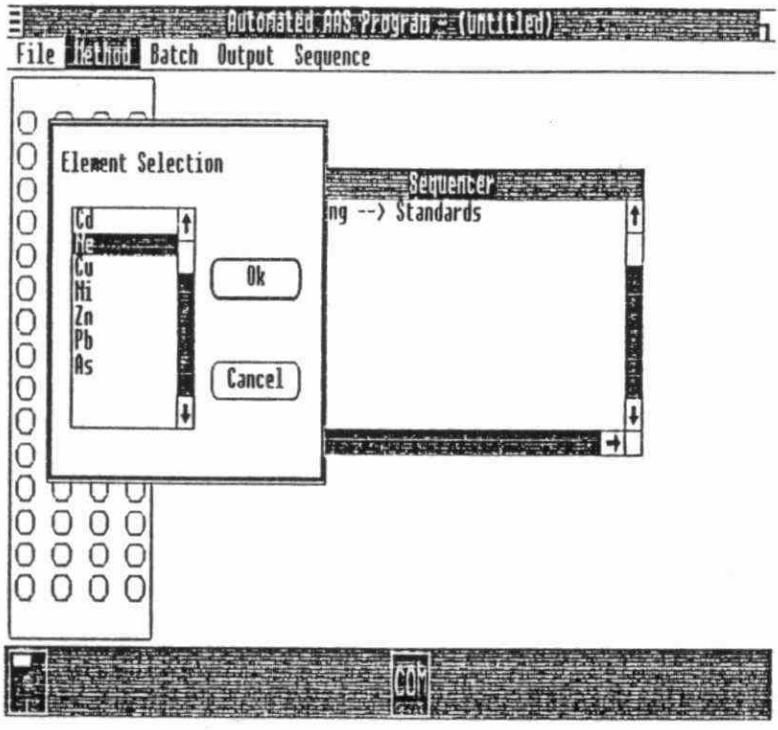
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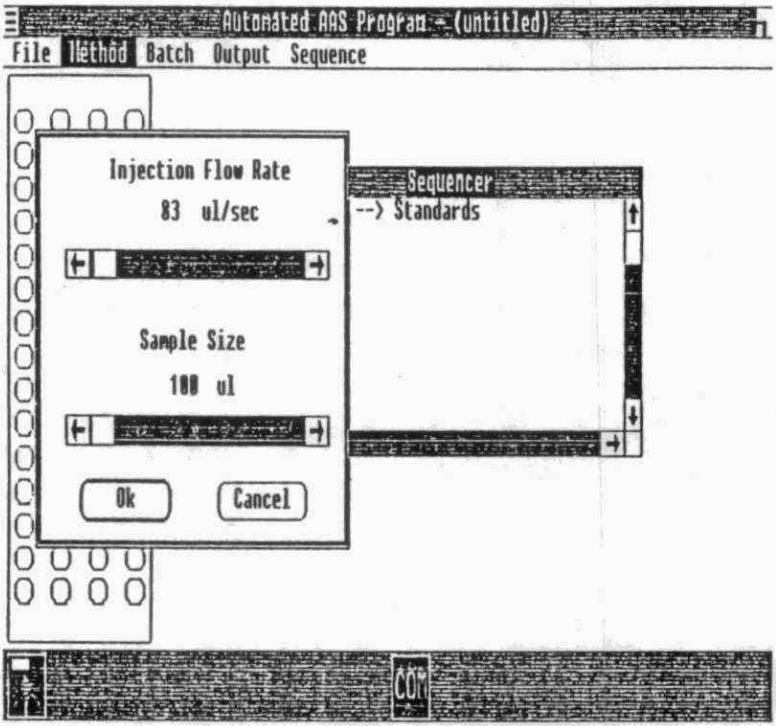
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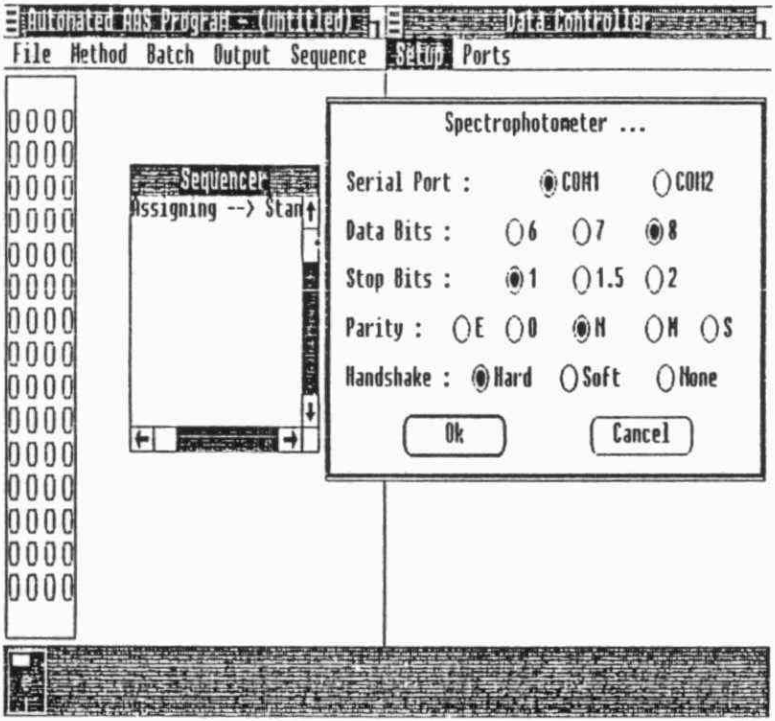
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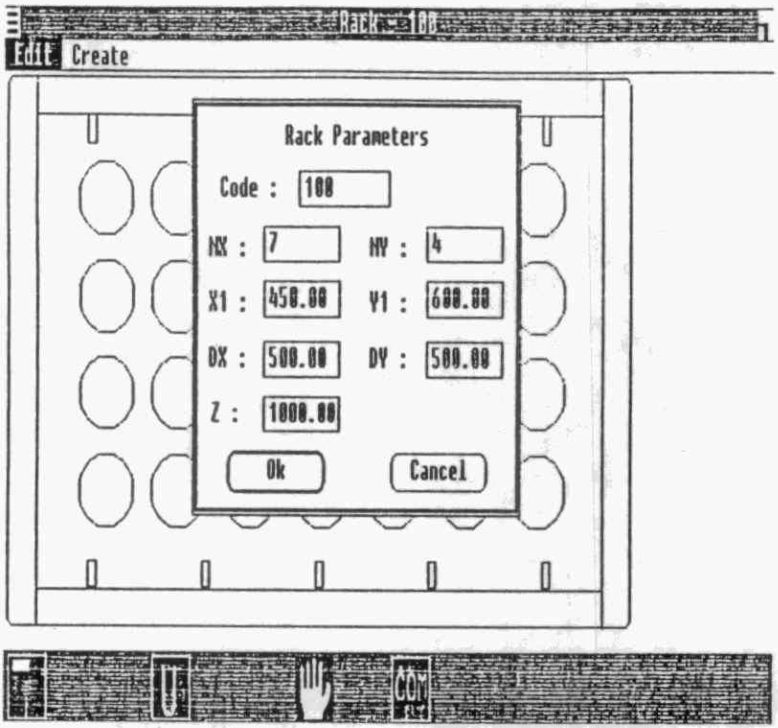
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SOLID-SUPPORTED ISOLATION AND DERIVATIZATION: AN APPROACH TO AUTOMATION OF ENVIRONMENTAL ANALYSIS

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Automation of analytical methodology is a means of reducing costs, decreasing time of analysis and improving precision. The instrumental and data handling aspects of analysis have been highly automated for some time now. Currently automation of the sample preparation phase of analytical methods is receiving attention. Solid phase sample preparation has attracted both academic and industrial interest as one approach to both simplification of manual methods and total automation.
In this context solid phase sample preparation refers to isolation and purification steps involved in carrying a sample through to quantitative determination of analyte.

In high sensitivity analyses, however, simple isolation and derivatization is frequently insufficient preparation of analyte and analytical derivatization is either preferred or obligatory. In such instances it has not been possible to take advantage of solid phase techniques since analytical derivatizations have as a rule been carried out in solution. We have reported a class of solid supported reactions that allow simultaneous adsorption of organic acids from water and derivatization to products detectable at high sensitivity by Gas or High Pressure Liquid Chromatography. The purpose of this investigation is to apply such reactions to the development complete solid supported sample preparation techniques for environmental analyses.

Determination of organic analytes in the environment is an area where both automation and derivatization play a major role. Derivatization with halogenated (usually fluorine) reagents is carried out to increase sensitivity of detection for organic acids such as the chlorophenoxy acetic acid herbicides or phenols. In such studies, as with many other environmental investigations, analysis of many samples for determination of very low concentrations of analytes is a common requirement.

Several factors must be considered given current technology. The first is that most analytical derivatizations are carried out in solution and this makes subsequent automation relatively difficult. The second is that the catalysts used in such derivatizations are highly toxic. For instance the Federal Register of the United States describes procedures for organic determining acids from environmental samples using crown ethers as a catalyst for derivatization of these analytes with PFBBr. The reagent is both an alkylating compound which should in principle be carcinogenic and a very potent lachrymator. Despite this the Register procedures carry repeated warnings of toxicity of the crown ethers use as phase transfer catalysts listing the

reagent simply as noxious. Thus current technology carries with it both technical problems for development of automation was well as considerable occupational hazard.

We propose to investigate solid supported reactions on XAD-2 as an approach to achieving the following objectives:

(i) To establish a complete solid phase sample preparation method including isolation, derivatizations and purification;

(ii) To apply this technology to the development of simplified and safer manual methods for analysis of organic acids from environmental samples;

(iii) To develop a robotic based sample preparation scheme based on solid phase sample preparation techniques.

The use of solid supports in purification of sample extracts have been reasonably well investigated and it was our belief that these methods can be incorporated into new techniques with relatively little modification. In contrast the reactions on solid support of resins such as XAD-2 have only recently been reported and the technology itself requires further study. Accordingly a primary focus was identification of reaction conditions that achieve optimum yield and that are consistent with the requirements of analytical methodology. Moreover, it was taken as a given that any reaction /work-up conditions that developed should also be such that they would be incorporated into a general sample preparation scheme.

The model for the envrinonmental investigations was 2,4 Dichlorophenoxy acetic acid. Initial studies with this analyte indicated that the reaction was feasible but that there was a major drawback: derivatization required 50 uL of PFBBr impregnated on 200 mG resin. This amount of reagent produced reaction product but also considerable background interferences. It was thus an immediate priority for reasons of cost as well as reduction of interferences to reduce the amount of that reagent required to obtain a reasonable yield of the desired PFB ester. Our previous work with other organic acids provided guidelines.

Determinants affecting reaction yield for the derivatization of acids from aqueous matrix have been established in our laboratory and are summarized in Table 1. It is perhaps not surprising that for some of these determinants reaction yield varies as the adsorption efficiency as it is reasonable to assume that the reagent and the analyte must be present in the same phase in order to react. Thus increase in amount of resin and the concomitant increase in surface area results in an increased reaction yield. Similarly treating the hydrophobic resin with a water soluble organic solvent to enhance wetting of the surface also increases the yield. Furthermore if the PFBBr was added onto the resin using techniques which would produce a more homogeneous distribution of reagent then the reaction rate for

the analyte increased. In addition polar compounds such as prostaglandins which are water soluble di or trihydroxy carboxylic acids reacted at a slower rate than lipophilic analytes. It was not clear however if the reduction in reaction yield with increased polarity was due to this physical characteristic or if the structurally complex nature of the analyte was the determining factor.

TABLE 1: THE EFFECT OF ANALYTE STRUCTURE AND IMPREGNATION TECHNIQUE ON REACTION YIELD.

Carboxylic Acid	Impregnation Technique	Volume PFBBr used	Reaction Conditions (Temp./Time)	Yield %+/rsd n=6
pentadecanoic	Dropwise Addition[1]	50	25°C/45min	75+/-7
	Freon Deposition[2]	10	30°C/20min	64+/-5
		15	30°C/20min	76+/-5
	Addition			
	(a) neat[3]	10	30°C/20min	32+/-2
	(b) in 100 uL TCE solution[4]	10	30°C/20min	67+/-8
Prostaglandin $F_{2\alpha}$	Addition			
	(a) neat[3]	10	40°C/120min	48+/-8
	(b)in TCE solution[4]	10	40°C/120min	90+/-9
	(c) in HEX solution[4]	10	40°C/120min	95+/-7
2,4-Dichloro-phenoxy acetic acid	Addition in TCE solution	10	30°C/30min	25+/30
		10	40°C/120min	90+/-8

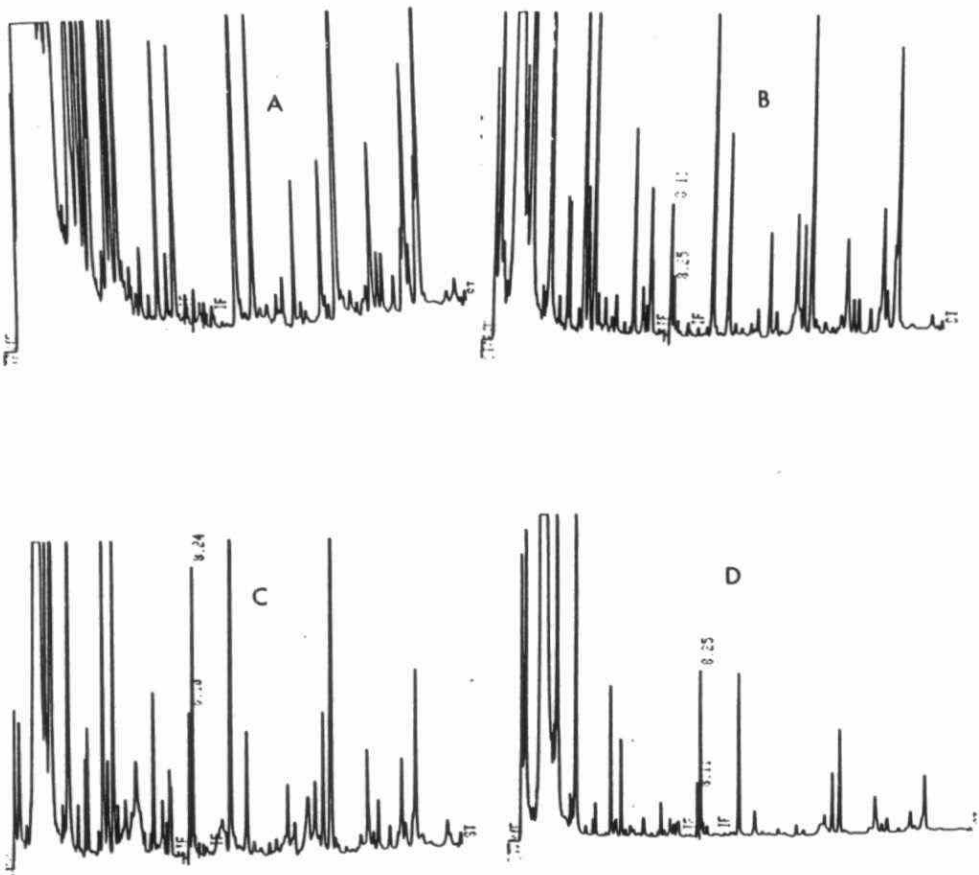
(1) 50 uL PFBBr added dropwise to 200 mG of resin
(2) A volume of PFBBr was dissolved in 4 mL of Freon 11 (B.P. 27°C) which was added to 200 mG resin. The solvent was allowed to evaporate at 30°C.
(3) Ten uL PFBBr was added directly to a mixture of 200 mG resin in 4 mL phosphate buffer at pH 7.4.
(4) Ten uL PFBBr in 90 uL 1,1,2 Trichloroethylene (TCE) or Hexane (HEX) was added directly to a mixture of 200 mG resin in 4 mL phosphate buffer at pH 7.4.

Studies on derivatization of 2,4 Dichlorophenoxy acetic acid showed that reaction rate for pentafluorbenzylation of this analyte was considerably slower than for the lipophilic acids used in the model investigations. Reaction times to produce high yield were longer and the temperature required was higher. Although 2,4 Dichlorophenoxy acetic acid has no free hydroxyl groups and is a relatively simple structure the reaction profile for this analyte was similar to that of the prostaglandins. It thus appears that the polarity of the compound rather than structural complexity are the major determinants of reaction rate on XAD-2. This is encouraging as it appears that we may be able to replace TCE with hexane as the solvent used to dilute the PFBBr for derivatization of 2,4-Dichlorophenoxy acetic acid.

It was anticipated that the reaction mixture would be complex as there is considerable information in the literature that glassware is contaminated to a level of 200 -300 nG of intermediate to long chain carboxylic acids per reaction tube. It was reasonable to assume that environmental samples must also be similarly contaminated and very probably to a greater extent. Accordingly we set the objective of incorporating a clean-up step in the procedure. This could be simply affected by linking the 200 mG reactor bed to a 4 mL mini-column of florosil (prepared in-house). The linked reactor bed/ chromatographic column system was washed with hexane. The first 2 mL effluent (Figure 1A) removed a significant amount of interferences with no detectable PFB-2,4 Dichlorophenoxy acetate being eluted. The second 2 mL of effluent removed additional interferences with approximately 5 % of the 2,4-Dichlorophenoxy acetate eluted. The next two fractions of 2 mL each removed approximately 80 % of the derivatized analyte. Subsequent washes contained only small traces of the of the desired product. It is clear that more investigation is required to provide even better clean-up procedures. Nevertheless it is also apparent that the approach of linked columns may provide a reasonable solution to the problem.

The areas that will be explored are improvements in separation technology and establishing a robotic based sample preparation system. Separation technology may be improved simply by increasing the amount of Florosil or decreasing the particle size to improve chromatographic efficiency. Alternatively the use of other phases such as silica gel or alumina will also be investigated. The reaction of 2,4-Dichlorophenoxy acetic acid when Hexane is used as a diluent will also be investigated in order to reduce the use of potentially harmful reagents. The peripherals to the robotic system have already been tested manually and appear to produce similar results as the method carried out using standard glass ware and sample handling techniques. Finally we intend to determine if the developed sample preparation techniques can reduce the detection limit from the present 5 nG/mL when 4 mL of water each containing 5 nG of 2,4-Dichlorophenoxy acetic acid are used in the reaction.

FIGURE 1: GAS CHROMATOGRAPHY/ELECTRON CAPTURE DETECTION TRACES FOR ISOLATES OBTAINED BY DERIVATIZATION OF 100 nG 2,4-DICHLOROPHENOXY ACETIC ACID FROM 4 mL BUFFER AT pH 7.4: A, B, C and D REPRESENT SUCCESSIVE 2 mL ELUANT FRACTIONS FROM THE REACTOR BED/CHROMATOGRAPHIC COLUMN LINK. PFB 2,4-DICHLOROPHENOXY ACETATE HAS A RETENTION TIME OF 8.25 MINUTES.



Preparation of Heterocyclic PAH's for Analytical Standards

E. Lee-Ruff , B. George, K. Kalamatianos and F. Ablenas, Dept. of Chemistry, York University, North York, Ontario M3J 1P3.

INTRODUCTION

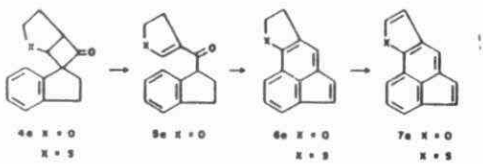
Recently, interest in the determination of heterocyclic polynuclear aromatic compounds in environmental samples has increased. A number of specific aza and sulphur PAH's have been identified and shown to exhibit potent mutagenic activity[1-5]. Polycyclic aromatic sulphur heterocycles have been reported in air particulate samples[6,7], carbon blacks[8-10], coal liquids[11,12], shale oil[12,13], coal tar[14] and fish[15]. Elemental analysis of petroleum has shown that it contains up to 6 percent sulphur[16]. The total sulphur in coal ranges from 0.2 to 11 percent and most of the sulphur is incorporated in the form of organic sulphur polycyclics such as thiophenes. Unlike the carbocyclic PAH's, the routine identification of sulphur and aza aromatic polycyclic derivatives and their biological activity studies have been limited primarily due to a lack of reference standards.

RESULTS AND DISCUSSION

We have developed a program for the expedient synthesis of novel oxygen and sulphur heterocyclic aromatic derivatives and the provision of these as analytical standards to the MOE. The program involves the development of two methods illustrated in Figures 1 and 2. The first of these employs the strained cyclobutanones as intermediates and the observation of regiospecific ring-opening reactions under acidic and basic conditions. Regiospecific α-ring opening takes under a variety of acidic conditions. This is postceded by ortho ring annelation in the same step and the formation of polycyclic ketones which can be structurally elaborated to the angular fused polycyclic. On the other hand, base reaction of the cyclobutanones proceeds with β-ring opening with formation of the carboxylic acid derivatives which can be recyclized to the α-aryl substituent giving the linear-fused polycyclic ketones which are readily converted to the linear fused polycyclics. The heterocyclic ring is incorporated either as part of the bicyclic ketone ring system (**X**) or as part of the α-aryl substituent (**Y**). The advantage of this method is the use of a single intermediate, readily available from cycloadditions of ketenes with the corresponding olefins, in the divergent preparation of both linear and angular fused heterocyclic aromatic derivatives in an expedient fashion.

The second approach involves the cyclizations of arylmethyl cations bearing an α carbonyl or thiocarbonyl groups (Figure 2). For the thiocarbonyl subststituted derivatives exlusive cyclization takes place with formation of PAH's incorporating a terminal thiophene ring.

The first approach has been utilized in the synthesis of furan and thiophene aromatic polycylics related to acephenanthrylene[17]. The bridged furan and thiophene derivatives 7a are prepared from the cylobutanones



4a X = O
X = S

5a X = O

6a X = O
X = S

7a X = O
X = S

4a available from the cycloaddition of indenyl ketene and dihydrofuran and dihydrothiophene respectively. Upon treatment of these ketones with polyphosphoric acid dehydration by way of the ring-opened intermediate 5a to give the dihydro-derivatives 6a was observed. These were efficiently dehydrogenated to the desired aromatic compounds 7a with DDQ. The UV spectra of 7a were similar to that of acephenanthrylene. Confirmation of the structural assignments was obtained from the high field proton and carbon-13 NMR spectra of these compounds.

Recently we have observed that the reduced cyclobutanols bearing an α-2 or 3-thienyl group undergo regiospecific ring-opening reactions under acidic conditions, an unprecedented result[18] (see Figure 2). These observations have now been extended to include the preparation of the linear thiophene analogues 2 and 3 of the mutagenic aceanthrylene 1 (Figure 3). The approach involves the use of the α-thienyl cyclobutanones incorporating a two carbon α-methoxyethyl group. Reduction to the cyclobutanol takes place in 90% yield with LAH. Subjecting these alcohols to a 2% ethanolic solution of H_2SO_4 results in dehydration and rearrangement to the tetrahydronaphthalene derivatives shown in Figure 3. Dehydrogenation of the tetrahydronaphthalenes gives the corresponding aromatic compounds and reaction of the methyl ethers with hydrogen iodide transforms these to the corresponding alcohols. Cyclization of the two carbon bridge to 2 and 3 is in progress.

More recently we have used the second approach in the preparation of PAH's possessing a terminal thiophene ring. The α-hydroxythioamides **8-11** (see Figure 4) undergo dehydration in acid to give the thiophenes **12-15** respectively. Experiments are currently underway to modify the α-hydroxythioamides in order to eliminate the N,N-dimethylamino substituent.

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FIGURE 1

SYNTHESIS OF PAH'S WITH NON-BENZENOID RINGS

Cyclobutanone Route

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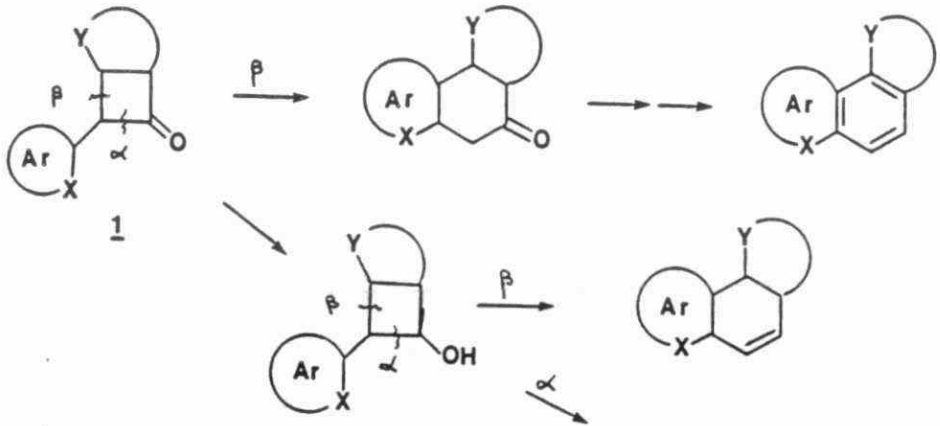


Linear-fused;

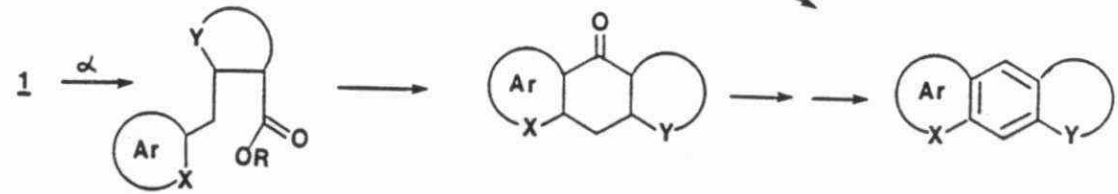
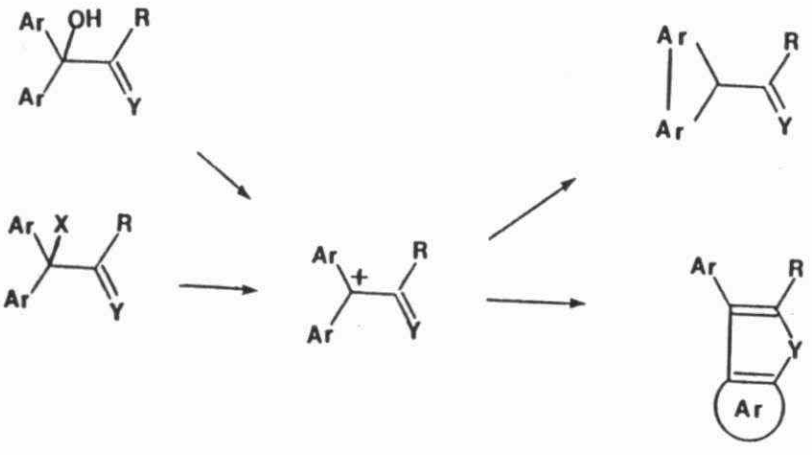


FIGURE 2

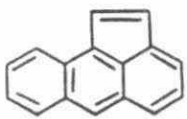
SYNTHESIS OF PAH'S WITH NON-BENZENOID RINGS

Arylmethyl Cation Route



Y = O, S

FIGURE 3



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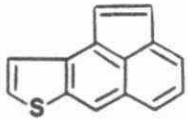

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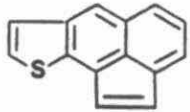

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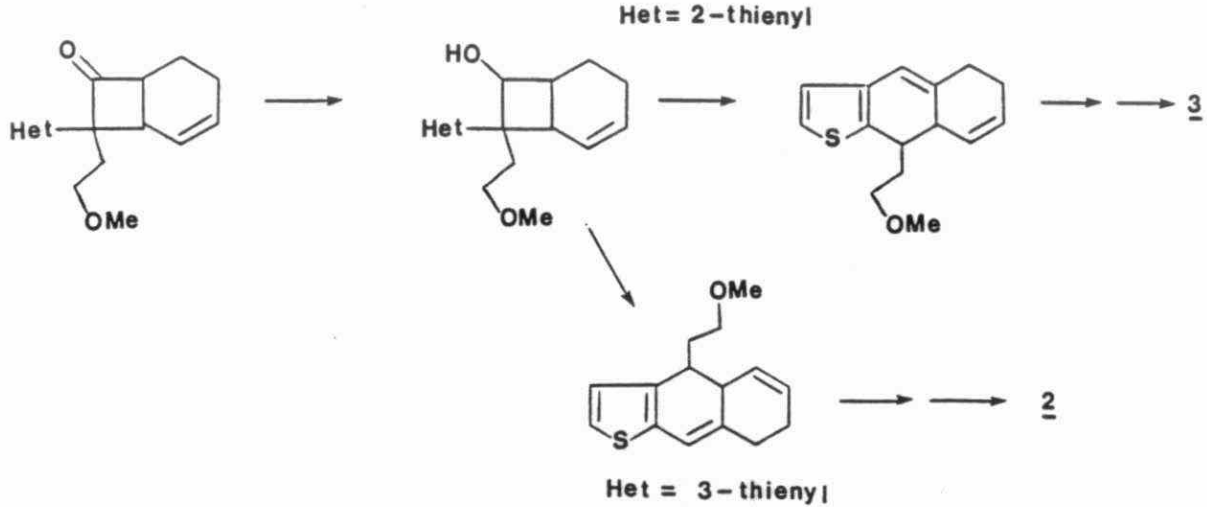

FIGURE 4

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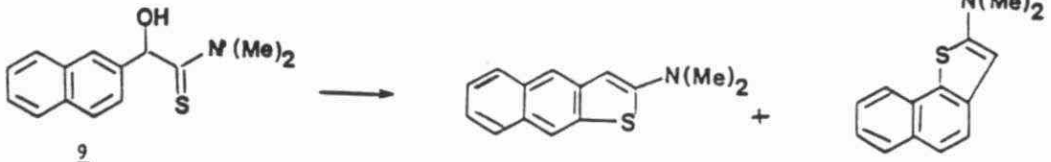

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TARGETED SYNTHESES OF SEVERAL CLASSES OF ISOMERICALLY PURE PAHS

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INTRODUCTION

Since the initiation of monitoring in the late 1950's, PAHs have been detected world wide in trace quantities in airborne particulate matter, water, soil and sediment, industrial and solid waste, work-place atmosphere, raw and processed foods, and tobacco smoke (1). Intense interest in PAH environmental research continues unabated as new pollutants are detected, their carcinogenic nature established, and structure-biological activity studies undertaken in order to understand and hopefully to combat their adverse effects (2).

In support of these investigations, synthesis and complete physicochemical characterization of PAHs is a *sine qua non* endeavor. In general, synthetic work leading to the provision of analytical standards of various PAHs has not kept pace with the sophisticated methodology used in modern quantitation and validation of PAH samples. With the exception of studies by Newman (3), Harvey (4), Muschik (5), and LaVoie (6), most preparations of PAHs have involved classical methods which lack generality, efficiency, and brevity. Additional shortcomings of these routes which cannot be ignored in view of modern knowledge are a) the necessity to handle potentially carcinogenic intermediates in significant quantities over many synthetic steps and b) the uncertainty of purity of final PAH products which have passed through synthetic stages producing isomeric products.

In order to overcome these shortcomings, we have been engaged in the preparation and provision of a variety of PAHs as pure analytical standards for environmental research. To date, we have prepared a significant number of PAH samples of the following structural types: a) anthracene and polycondensed anthracene PAHs and their corresponding quinones, b) methyl-PAHs and methyl-PAH quinones, e.g. 8-methyl-benz[a]anthracene, c) bromo- and chloro-PAHs, and d) aza-PAHs, e.g. benz[a]acridine and benz[c]acridine(7).

Our approach to PAH synthesis has always subscribed to the following credo: "Often a multistep synthesis route which ensures formation of a single isomer is preferred to separation of a minor component from a complex mixture" (8). Furthermore, we subscribe to the following guidelines for our synthetic objectives:

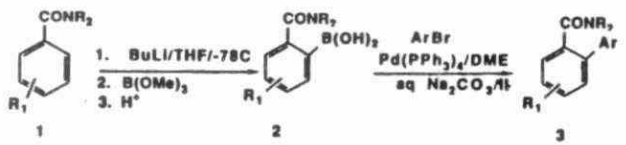
a) the majority of routes should be accomplished in a minimum number of steps (usually 5-6 steps) starting from commercially available materials. The obvious advantage is the avoidance of excessive handling of potentially dangerous materials,
b) the key step(s) should have precedence and experience in our laboratories,
c) the order of priorities should be established according to environmental research requirements.

In the continuation of our synthetic PAH work, we have undertaken the regioselective preparation of three classes of compounds (phenanthrene, phenanthridine, nitrofluorene, and nitrofluoranthene). These results constitute the subject of this report.

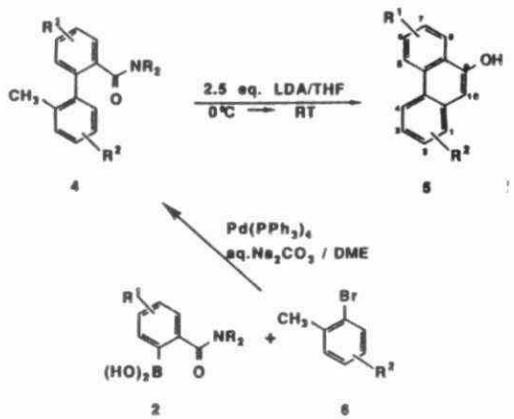
Synthesis of Phenanthrols and Phenanthrene PAHs

Phenanthrenes constitute a major class of PAHs derived from air, water, gasoline, diesel exhaust, tobacco smoke, and smoked foods (9). Although phenanthrene and its dihydro diols show no significant tumor-initiating properties, trimethyl and tetramethyl derivatives are found to be mutagenic in bacterial systems (10). Phenanthrene metabolism via dihydrodiols has been demonstrated in bacterial and mammalian systems (11). Historically, phenanthrene oxides were used in the first demonstration of epoxide hydrase activity on an arene oxide (11) and in early model studies to establish the nature of PAH ultimate carcinogens (12).

Synthetic approaches to the phenanthrene nucleus are based on classical Wagner-Meerwein ring expansion of fluorene derivatives (13), Pschorr synthesis (13,14), Ullmann reaction (15) and, more recently, on the Mallory photocyclization (13,16) methodology. For the synthesis of polysubstituted phenanthrenes, many of these methods are compromised in efficacy and brevity by the lack of regioselective routes to the requisite substituted benzene precursors.



Scheme 1



Scheme 2

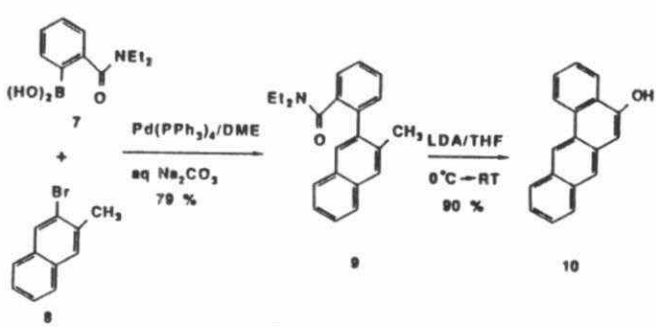
Recently, we reported a new protocol for the synthesis of biphenyls **3** using transition metal catalyzed cross coupling reactions between aryl boronic acids **2** and aryl bromides **1** **(Scheme 1)**(17). Application of this methodology has led to the preparation of 2-carboxamido-2'-methylbiphenyls **4** which upon treatment with lithium diisopropylamide (LDA) has been shown to give 9-phenanthrol derivatives **5** in good yields **(Scheme 2)**. In view of the availability of precursors **2** regiospecifically by directed ortho metalation (17) and o-bromotoluenes **6** by conventional means, the new phenanthrene synthesis has broad scope and advantage over many of the classical methodologies (13,14,15).

Table 1 lists the biphenyl precursors **4** which were prepared by the previously described procedure (17) and used in the synthesis of the corresponding phenanthrols. In a typical reaction, 2-N,N-diethyl carboxamido-2'-methylbiphenyl (**4**, R^1=R^2=H) was treated with LDA (2.5 equiv/THF/0° C RT over 3 h) gave 9-phenanthrol (**5**, R^1=R^2=H in high yield. Under similar conditions, the corresponding N,N-diisopropyl amide afforded 9-phenanthrol in even greater yield thus indicating the absence of operation of a steric effect in the overall cyclization process. A number of substituted biphenyls likewise led in good to excellent yields to phenanthrols bearing chloro, methoxy, and carboxamido substituents.

Table 1. Synthesis of Phenanthrols

Substituents		Yield, %	
R^1	R^2	4	5
	R = Et		
H	H	87	92
H	2-Cl	85	97
H	2-$CONEt_2$	77	78
6-OMe	2-Cl	43	89
5-OMe	H	23	92
8-OMe	H	85	92
H	2-NO_2	67	SM
	R = i-Pr		
H	H	55	98
H	3-NO_2	77	SM

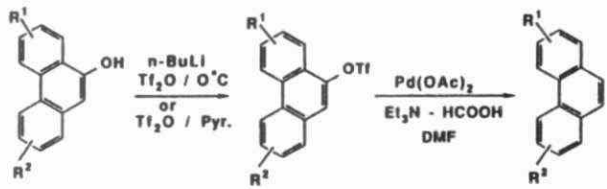
Applicability of our method to the preparation of more highly condensed PAHs was demonstrated by the synthesis of 5-hydroxy-benz[a]anthrancene **10** **(Scheme 3)**. The naphthylphenyl amide **9**, prepared by cross coupling of the boronic acid **7** with the bromonaphthalene **8**, was subjected to the LDA-mediated cyclization conditions to give **10** in high yield.



Scheme 3

Although nitro biphenyls were readily prepared by the cross coupling regimen **(Table 1)**, their cyclization under the LDA (up to 4 equiv/THF/reflux) or other basic conditions led only to the recovery of starting material. This may be due to the known propensity of nitroaromatics to undergo single electron transfer reactions with lithium amide bases (18). These observations do not allow, for the present time, to prepare nitrophenanthrene PAHs.

To demonstrate accessibility of the parent PAHs, two phenanthrols (**11**, $R^1 = R^2 = H$ and $R^1 = H$, $R^2 = Cl$) were converted into the corresponding phenanthrenes (**13**, $R^1 = R^2 = H$ and $R^1 = H$, $R^2 = Cl$) in good yields (50-60 %) via their triflate intermediates **12 (Scheme 4)**. In view of the demonstrated generality of this reductive method (19), other phenanthrols should be likewise amenable to conversion into the corresponding phenanthrene PAHs. Significantly, the 1- and 4-methoxyphenanthrenes are poorly accessible by previously described routes (20).



11 12 13

Scheme 4

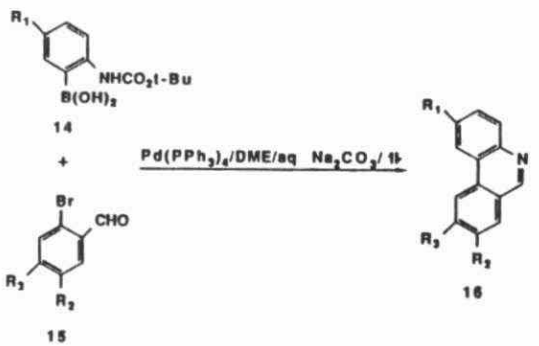
This general and efficient methodology is a useful addition to the preparatory routes available for phenanthrene PAH and, in many cases, has advantage in regioselectivity over more conventional procedures.

Synthesis of Phenanthridine PAHs

Phenanthridines are of long standing interest as pharmacologically active agents (21) and as subunits in several classes of alkaloids (22). In recent years, phenanthridine has been detected in environmental sources such as in wood preservative waste water, petroleum and diesel fuel (23). Evidence is accumulating that phenanthridine and some of its derivatives show mutagenic activity in **S. typhimurium** (Ames) test systems (24,25). Interestingly, a phenanthridinium betaine related to an alkaloid has been shown to exhibit antitumor properties (26).

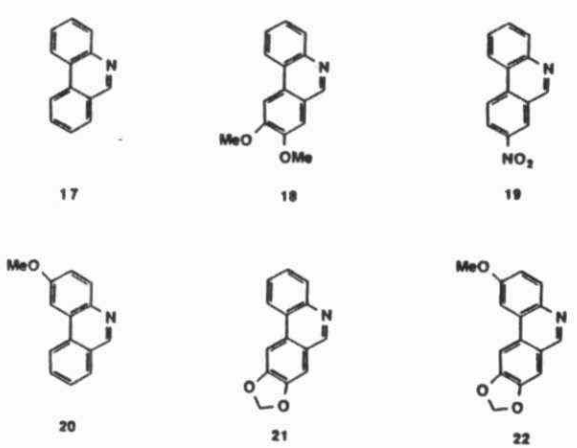
The major synthetic approaches to phenanthridines are of early vintage and include a) cyclization of 2- (Bischler-Napieralski) and 2,2'-substituted biphenyls, b) photochemical cyclizations of benzanilides and N-aryl benzamides, c) use of o-halo benzylanilines via benzyne intermediates, and d) Beckmann and Schmidt rearrangement of fluorenone and fluorenol precursors (27). Recently, phenanthridines have been prepared by remote metalation of 2-aminobiphenyls (28), and Pd-mediated cyclization of N-aryl benzamides (29).

We have been interested in establishing connections between the aromatic directed metalation reaction (30) and the transition metal catalyzed cross coupling methodology **(Scheme 1)**(17). As a further demonstration of this link, we have developed a new general route to phenanthridines **16** by cross coupling of the o-N-t-BOC aryl boronic acids **14** with o-bromobenzaldehydes **15 (Scheme 5)** (31). In view of the ready availability of compounds **14** by directed metalation-boronation of N-t-BOC anilines (17) and of the o-bromobenzaldehydes by classical methods, this represents an efficient and regiospecific route to phenanthridines which may have general utility.



Scheme 5

The results of our preliminary studies are summarized in **Scheme 6**. In a typical experiment, treatment of aryl boronic acid **14**, R^1 = H with o-bromobenzaldehyde **15**, R^2=R^3=H under Pd(0) catalysis as previously described (17) followed by trifluoroacetic acid mediated cyclization, leads directly to phenanthridine **17**, **(Scheme 6)** in good yield (74%). Similarly, a number of other aryl boronic acids **14** were coupled with bromobenzaldehydes **15** to give the corresponding phenanthridines **16 (Scheme 6)**. In some cases (18,21), cyclization occurred directly under the cross coupling conditions without the need for the trifluoroacetic acid step. Unoptimized yields vary from 50-90%.



Scheme 6

The results of these initial studies suggest that a number of substituted phenanthridine PAHs may be available in a regiospecific and short manner. This may be of value for the potential detection of new phenanthridine PAHs in the environment.

Synthesis of Nitrofluorene PAHs

Since 1978, evidence has been accumulating that nitro-PAHs are widely distributed in our environment, having been identified in urban ambient air particulates, diesel exhaust emissions, fly ash, photocopier fluids, and cigarette smoke (32). With the discovery, in 1980, of the potent direct acting mutagenicity of nitro-PAHs in microbial test systems (33), increasing attention has been devoted to their detection, identification, and their quantitation in the environment. To date, in excess of 200 nitro-PAHs have been recognized but carcinogenic-mutagenic activities for only a few have been established (32).

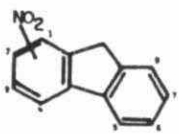
The rapid evolution of nitro-PAH research necessitates the availability of pure analytical standards in order that monitoring, quantitation, and biological and metabolic studies can proceed unabated. Of the numerous nitro-PAH types, the nitrofluorene (NO^2-F) and nitrofluoranthene (NO_2-Fl) classes constitute the most potent "direct-acting" mutagens whose detection, identification, and metabolism are under current intense investigation (34). We have therefore targeted our initial studies towards the rapid and convenient preparation of these classes of nitro-PAHs in order to provide them in high purity for the required analytical and biological environmental work.

The currently available methods for nitro-PAH preparation are mostly based on electrophilic nitration and other classical reactions which suffer from low yields, lack of isomer specificity, limited scope, and considerable inefficiency (8). The synthetic routes to NO_2-F and NO_2-Fl are no exceptions to this analysis. Although in some cases direct nitration leads to a major NO_2-F and NO_2-Fl isomer which is readily isolated and purified, e.g. 2-NO_2-F and 3-NO_2-Fl respectively, the derivation of any other NO_2 isomers usually requires a) detailed investigation of reaction conditions to obtain them in significant amounts in a mixture and b) tedious purification to achieve the 99.5% or better purity demanded for analytical work.

As a further extension of the cross coupling methodology for the synthesis of unsymmetrical biaryls discovered in our laboratory (**Scheme 1**)(17), we are engaged in developing a regiospecific route to NO_2-F and NO_2-Fl derivatives and their metabolic products. The potential scope of our investigation, indicated in **Table 2**, is designed to provide all possible NO_2-F and NO_2-Fl isomers and many of their possible metabolites as pure compounds without contamination of related NO_2 derivatives.

Table 2:

Provision of Nitrofluorenes and Nitrofluoranthenes and their Hydroxylated Derivatives.

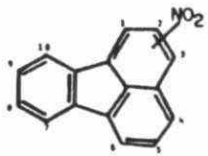


Nitrofluorene(F)

- 1-NO_2-F
- 2-NO_2-F
- 3-NO_2-F
- 4-NO_2-F

Metabolites/Oxid. Products:

- 1-NO_2-5-OH-F
- 2-NO_2-5-OH-F
- 3-NO_2-5-OH-F
- 4-NO_2-5-OH-F
- 1-NO_2-6-OH-F*
- 2-NO_2-6-OH-F*
- 3-NO_2-6-OH-F*
- 4-NO_2-6-OH-F*
- 1-NO_2-7-OH-F
- 2-NO_2-7-OH-F
- 3-NO_2-7-OH-F
- 4-NO_2-7-OH-F



Nitrofluoranthene (FA)

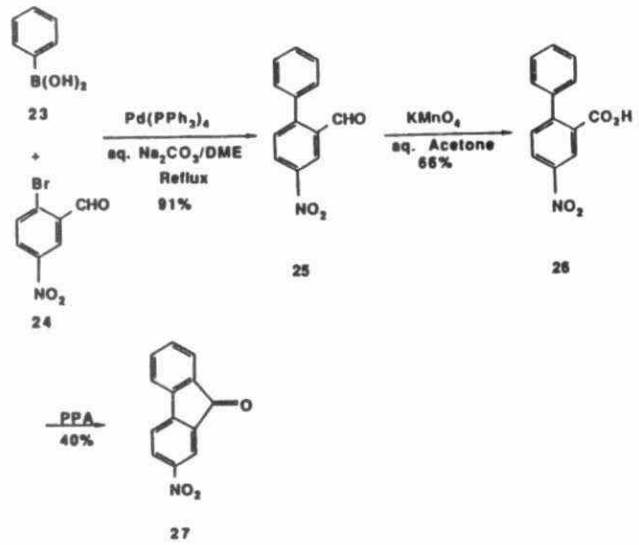
- 1-NO_2-FA
- 2-NO_2-FA
- 3-NO_2-FA
- 7-NO_2-FA
- 8-NO_2-FA
- 9-NO_2-FA
- 10-NO_2-FA
- 3,7-diNO_2-FA
- 3,8-diNO_2-FA
- 3,9-diNO_2-FA
- 3,10-diNO_2-FA

Metabolites/Oxid. Products:

- 3-NO_2-6-OH-FA
- 3-NO_2-8-OH-FA(?)
- All isomeric 1-, 2-, 3-, and 4-NO_2-FA with 5-, 6-, and 7-OH available from corresponding NO_2-OMe-F precursors.

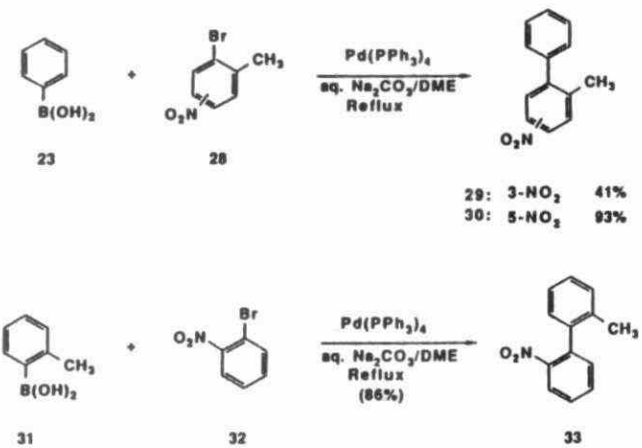
Priorities for the synthetic work are being established in consultation with the Ministry. Initially, the preparation of all of the four NO_2-F isomers is being undertaken in order to a) establish the viability and generality of the route, b) provide those NO_2-F isomers which are poorly accessible and/or not commercially available (1-, 2-, 3-NO_2-F), and c) obtain sufficient materials of all NO_2-F isomers for use as intermediates in our proposed preparation of the NO_2-Fl derivatives.

As a model test of viability, we have recently completed the synthesis of 2-NO_2-fluorenone **27** (**Scheme 7**). Thus application of our transition metal catalyzed cross coupling procedure (**Scheme 1**) to phenyl boronic acid **23** with the bromo nitrobenzaldehyde **24**, easily prepared by conventional chemistry, led, in excellent yield, to the biphenyl **25**. Potassium permanganate oxidation of **25** under standard conditions gave the corresponding carboxylic acid **26** whose polyphosphoric mediated cyclization provided the nitrofluorenone **27**. Although the overall yield is reasonable, the yields of the various steps have not yet been optimized. The reduction of **27** into the target 2- NO_2-F derivative is under current investigation.



Scheme 7

The successful model study in hand, we have pursued the preparation of nitro-biphenyl precursors for the other, highly inaccessible NO_2-F isomers. Starting with readily available aromatic precursors, the preparation of the requisite nitro-biphenyls has been achieved (**Scheme 8**). Thus the 3- and 5-NO_2 biphenyls, **29** and **30**, representing precursors for the 1- and 3-NO_2-fluorenones respectively, have been prepared by cross coupling of phenyl boronic acid **23** with the corresponding 2-bromo-6-nitrotoluene and 2-bromo-4-nitrotoluene **28**. The latter substances are available from commercial sources. Similarly, the 2-methyl-2'-nitro biphenyl **33**, the penultimate precursor for the 4-NO_2-F isomer, has been obtained in high yield by cross coupling of o-tolyl boronic acid **31** with o-bromonitrobenzene **32**.



Scheme 8

Experiments to convert compounds **29**, **30**, and **33** into the corresponding carboxylic acids by standard oxidation of the tolyl methyl groups are in hand. The further Friedel-Crafts cyclization and ketone reduction of these systems are well precedented. It is therefore anticipated that all theoretically possible NO_2-F isomers will be accessible in high purity and preparative quantity for use as analytical standards and as intermediates towards the synthesis of the NO_2-Fl systems according to established chemistry (32).

ACKNOWLEDGEMENT

We are grateful for the continuing support of our synthetic programs on PAH by the Ontario Ministry of the Environment.

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SYNTHESIS OF CHLORINATED ANILINES

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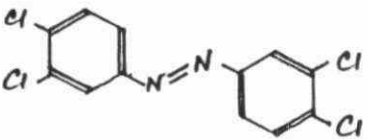
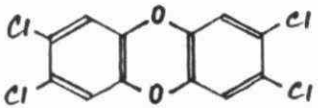
The toxic compound 3,3',4,4'-tetrachloroazobenzene (TCAB) has been found as a contaminant in certain commercial herbicide preparations. The azo compound is very persistent in the environment and has been recovered from soil samples treated with the subject herbicides. Of particular concern is the similarity in molecular shape and also toxicological properties of TCAB to the highly toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).



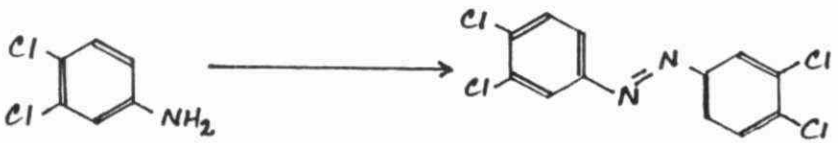
TCAB



TCDD

In this project, reference samples of a series of symmetrically chlorinated azobenzenes were prepared by oxidation of the corresponding chloroanilines. A modification of a published procedure for oxidation was developed, in which silver carbonate on Celite was the oxidizing agent. Repeated chromatography was needed to obtain pure products.

e.g.



Since completing this short project for MOE we have synthesized a large number of additional substituted azobenzenes. These compounds have been studied in a competitive assay versus TCDD for binding to the hepatic Ah receptor protein from the male Wistar rat. A complete table of these results, expressed as EC_{50} values for displacing radiolabelled TCDD from the Ah receptor, is presented in the poster.

DUAL CAPILLARY COLUMN ANALYSIS OF POLYCHLORINATED BIPHENYLS (PCBs) IN DRINKING WATERS.

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Increased public awareness of the affects of chronic exposure to PCBs has sparked the need for fast accurate methods for quantitating PCBs in environmental samples. Original Ministry of the Environment quantitation methods involved solvent extraction followed by either perchlorination of all biphenyl to decachlorobiphenyl and its subsequent analysis or the quantitation and grouping of PCB peaks generated by single packed column Gas Chromatographic (GC) analysis. The advent of capillary columns allowed for much greater resolution of individual PCB isomers and more accurate quantitation. Further enhancement of PCB quantitation techniques has been accomplished by using dual fused silica capillary column methodology for quantitation and confirmation of results. Dual capillary column PCB quantitation methodology for the analysis of drinking water and drinking water sources was developed using PCB standards and fortified water samples.

Wet chemistry preparation of samples for GC analysis was similar to that employed for organochlorine pesticides and consisted of liquid/liquid extraction, concentration by rotovapor apparatus, wet pack florisil cleanup and final concentration by vortex evaporation. Sample extracts containing organic chlorine pesticides such as DDT and DDD along with PCBs were resplit using dry pack florisil cleanup and reanalysed.

Analytical methods were developed using a Varian 6000 Gas Chromatograph, equipped with a split/splitless injector and dual Electron Capture Detectors (ECD), coupled with a Varian Vista 402 data station. Sample extracts, split on injection, travel through two 30m 0.25 mm ID fused silica capillary columns of differring polarity (DB1/DB1701) before passing through the ECD detectors.

Two dual capillary column methods differing only in instrument calibration mixture were tested. Calibration mixtures containing Aroclors 1254 and 1260 were chosen because >95% of PCB positive samples received by the Drinking Water Section Organic Water Unit contain these Aroclors.

Method PCBMIX, using A 1:1 mixture of Aroclors 1254 and 1260 for calibration, was determined to be better than method PCB using Aroclor 1254 only. Method PCBMIX was much better for quantitating mixed PCB extracts although neither method was adequate when quantitating extracts containing large proportions of Aroclors other than 1254 and 1260. Instrument reproducibility using method PCBMIX was 3% with a linear range of 20 to 500 ng/ml. Spike recoveries averaged 107% ± 8% over the 20 ng/l to 2000 ng/l range tested.

Method PCBMIX was checked and validated against current Ministry of the Environment single packed column PCB quantitation methodology using laboratory standards and fortified water samples as well as real environmental drinking water samples. Results obtained were comparable. No significant difference was found between the quantitation methods although method PCBMIX results tended to be on average slightly higher.

A COMPARISON OF MASS SPECTRAL INSTRUMENTAL CAPABILITIES (LRMS, HRMS & MS-MS) FOR CHLORINATED DIBENZO-P-DIOXIN AND DIBENZOFURAN DETERMINATION

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Ultra-trace determinations of chlorinated dibenzo-p-dioxins (CDDs) and dibenzofurans (CDFs) in environmental samples are performed using the technique of gas chromatography - mass spectrometry (GC-MS). Many different GC-MS techniques have been employed, including the use of various types of mass analyzers. The three analyzers that have been most extensively investigated include low resolution quadrupole (GC-LRMS) systems; high resolution, double-focussing systems (GC-HRMS); and tandem mass spectrometer systems (GC-MS-MS) based upon a dual quadrupole and collision cell (CAD cell) design.

The capabilities of the various GC-MS systems available for CDD/CDF determination are not the same. However, there is no single system that is best suited for all applications. The advantages and disadvantages of each type of system are summarized in Table 1. Previous studies have shown some of the differences that exist between certain instrument types for the analysis of real samples (1,2). In a joint study carried out by the Ministry of the Environment and SCIEX a comparison of GC-LRMS and GC-MS-MS analysis of fly-ash was made. It was shown that interferences detected by GC-LRMS were not detected in the GC-MS-MS data and it was also noted that the quantitative results obtained by GC-MS were consistently higher than those derived by

GC-MS-MS (1). Recently Clement et al (2) compared the instrument sensitivities of GC-LRMS, GC-HRMS and GC-MS-MS and found the HRMS and MS-MS techniques produced comparable sensitivities for the determination of 2,3,7,8-TCDD, while all three techniques were comparable for the determination of OCDD. (See Table 2)

To date, no systematic comparison of the capabilities of GC-LRMS, GC-HRMS and GC-MS-MS for CDD/CDF determinations has been reported. Such studies are needed to define the best area of application for each technique. The study to be presented will provide a comparison of the capabilities of each instrument type for the determination of CDD/CDF's as well as determine the linear dynamic range and the detection limits of each instrument type. The instrumental systems to be used during this study include a Finnigan 4500 (LRMS); a Hewlett Packard MSD (LRMS); a VG-ZAB-HS double focusing instrument (HRMS) and a Finnigan TSQ-70 MS-MS system.

During the initial stages of this study CDD and CDF standards at varying concentrations will be analyzed using selected ion monitoring to determine instrument linearity as well as detection limits. A comparison of real sample analysis will be carried out on fly-ash, in which fly-ash extracts at varying dilutions will be analyzed by each instrument type. The flyash extract will be prepared and diluted in a manner so as to represent detected levels of CDD/CDF above the detection limit, at the detection limit and below the detection limit of a LRMS instrument. A comparison of detected values, response factors and the presence or

absence of interferences will be made between each instrument type.

Preliminary work has indicated a marked difference in detection limits of different mass spectrometer techniques (Table 2). It has also been observed that the operating modes of certain instrument types affects the results obtained from CDD/CDF analysis (3). This phenomenon will also be investigated in this study.

TABLE 1: COMPARISON OF RELATIVE CAPABILITIES OF GC-LRMS, GC-HRMS AND GC-MS-MS

FEATURE	GC-LRMS	GC-HRMS	GC-MS-MS
SELECTIVITY	LOW	HIGH	MEDIUM
SENSITIVITY	MEDIUM	HIGH	HIGH
SAMPLE THROUGHPUT	HIGH	LOW	HIGH
UNIQUE FEATURES	---	EXACT MASS DETERMINATION	SPECIAL SCAN MODES
OPERATOR SKILL	LOW	HIGH	MEDIUM
COST OF EQUIPMENT	LOW	HIGH	HIGH

TABLE 2: RELATIVE SENSITIVITIES OF INSTRUMENTAL TECHNIQUES FOR CDD/CDF (2)

	AMOUNT INJECTED	OBSERVED SIGNAL : NOISE		
		LRMS	HRMS	MS-MS
2,3,7,8-TCDD	12 pg	10:1	125:1	100:1
OCDD	24 pg	15:1	25:1	15:1

References:

(1) Shushan, B., Tanner, S.D., Clement, R.E., & Bobbie, B., Chemosphere55, 14, 843-846 (1985)

(2) Clement, R.E., Bobbie, B., & Taguchi, V., Chemosphere, 15, 1147-1156 (1986)

(3) Ontario Ministry of the Environment, unpublished data.

ICP/MS Isotope Ratios: Identification of Atmospheric Emissions of Metals

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Identification of sources of pollutants is important for assessing environmental quality, and for planning and implementing abatement strategies. Reliable source identification at locations distant from the sources is usually difficult to achieve because of the large numbers of distinct sources emitting chemically-identical pollutants.

Analytical methods have been developed by the Ontario Ministry of the Environment which exploit slight differences in the isotopic composition of heavy metals from different sources to identify the fraction of these elements from each source. The results have been applied successfully to source apportionment of Pb in atmospheric aerosol samples from Dorset, Ontario, and a number of other locations. An exploratory study using Cd isotopes has also been carried out.

The approach required an analytical method with high sensitivity and specificity, low detection limits, and a low per sample cost. These conditions were satisfied by the use of ICP/MS (inductively-coupled plasma/mass spectrometry), which has detection limits on the order of 0.02 ppb for Pb. Method detection limits are actually determined by trace levels of metals in the sampling media, and are approximately 2.0 ppb for Pb, equivalent to 0.05 ng-m-3 of Pb in a 24-hour aerosol sample.

Most of the work to date has focused on the use of the 206Pb/207Pb isotope ratio in atmospheric samples. Because of a fortuitous combination of geological and economic factors, there is a significant difference in this ratio in Pb from United States and Canadian automotive exhaust, which remains the largest source (60% - 70%) of Pb in the North American environment. The 206Pb/207Pb ratio is determined by the geological age of the ore body from which the Pb was extracted. Pb compounds are added to automotive fuel as anti-knocking agents, which are eventually released into the atmosphere as combustion products. Primary Pb

sources for Canadian refineries are located in British Columbia and New Brunswick, with 206Pb/207Pb ratios around 1.15, while US refineries use lead ores with 206Pb/207Pb ratios in the range 1.20-1.25.

Aerosol samples collected in various Ontario and US cities around the Great Lakes confirmed the validity of the distinct ratios. Further proof was obtained from analysis of daily aerosol samples collected at Dorset by the Atmospheric Environment Service of Canada in the autumn of 1984 and the spring of 1986. The Lagrangian air parcel back-trajectories associated with each sample showed that the observed 206Pb/207Pb ratios were consistent with the likely sources of the atmsopheric Pb.

A simple receptor model was devised which used the mean 206Pb/207Pb source ratios to calculate the concentration-weighted fraction of Pb from US and Canadian sources. Approximately 46% of the atmospheric Pb at Dorset in 1984 came from US sources, with 52% from Canadian sources. Stratification of the data further revealed that episodes of high Pb concentrations were due to advection of pollutants from the United States. Observations of unusually low 206Pb/207Pb ratios (<1.15) in the Dorset samples were attributed to emissions from non-ferrous smelters in the Sudbury and/or Noranda Val d'Or regions. These sources accounted for about 2% of the atmospheric Pb observed at Dorset in the autumn of 1984.

Analysis of the 112Cd/114Cd ratio in the 1986 series of Dorset aerosol samples revealed a variability in the ratio which could not be explained by sampling or analytical variability. Analysis of Cd isotope ratios is complicated by isobaric and oxide interferences; however, the magnitude of these interferences can be calculated and corrections applied. A more serious limitation is the apparent lack of a simple correlation between the Cd isotope ratio and source type or region, as there is for Pb.

Further applications of the Pb isotope apportionment method are being considered, both for routine monitoring purposes and for application to other environmental matrices. Sources of other heavy metals, such as Cd and Hg, may also be identified eventually by their isotopic composition, but these will require further development of the methodology and a better definition of the conditions (eg., local- or regional-scale problems) when they might be employed most successfully.

A ROUTINE METHOD FOR ELEMENTAL ANALYSIS OF DRINKING WATER BY ICP-MS

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The analysis of Ontario's drinking water is one of the major responsibilities of the Ministry. Monitoring of elemental concentrations in drinking water is necessary to protect the public. The data is then used by environmental scientists for various studies.

As information concerning affects of different chemical compounds on the environment grows, so does the need to measure analyte concentrations at lower levels.

Many analytical methods have been developed in our laboratory using different instrumentation to measure elemental concentration levels in drinking water samples. In the past this instrumentation provided sufficient analyte detection capability.

Today there is a demand for lower detection limits. To achieve this many of the incoming samples are preconcentrated to gain sufficient analyte detection.

Inductively coupled plasma mass spectrometry (ICP-MS) is a relatively new technique which has proven its detection capability for many elements in the parts per trillion level. Using this technique for analysis of drinking water samples allows the elimination of a time consuming preconcentration step and simplifies analysis by reducing the number of analytical methods used.

Elemental concentrations measured with the ICP-MS are Be, B, Al, Ti, V, Cr, Mn, Fe, Ni, Co, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sb, Ba, Tl, Pb, and U. Detection limits are <.1 ng/ml for most elements.

The effects of spectral interferences have been investigated and corrected.

Accuracy and precision (long and short term) measurements have been made.

Calcium and magnesium form refractory oxides in the source. At high concentrations this chemical interference or matrix effect can cause inaccuracy of analyte measurements. Ontario's drinking water has varying concentrations of Ca and Mg. A suitable correction procedure has been incorporated into the method to eliminate this affect.

An instrument comparison has been conducted between ICP-MS and existing instrumental methods (ICP-AES, GFAAS, Hydride-AAS, and Fluorescence).

A quality control protocol has been established for maintaining the integrity of the data.

Software applications have been written to automatically check the results for accuracy and to transfer the data to our laboratory information management system.

THE APPLICATION OF SOLID PHASE EXTRACTION TO THE ANALYSIS OF CHLOROPHENOLS AND PHENOXY-ACID HERBICIDES

BY: DR. WILLIAM G. CRAIG AND DR. C. DAVID HALL

The liquid/liquid extraction procedure currently used for the extraction of organics from water is expensive and labor intensive. An alternative to liquid/liquid extraction is a technique referred to as SPE: solid phase extraction. This procedure involves a water sample passing directly through a modified silica column. The column is dried and eluted with an organic solvent. Thus, the extraction, concentration, and cleanup are accomplished in one step. If this technique could be applied to environmental samples it would greatly improve the cost effectiveness of the analysis. A joint Ministry of the Environment/Paracel Laboratories research project was initiated to investigate thed application of solid phase extraction to the extraction of chlorophenols and phenoxy acids from water.

The study consisted of six parts:

1. A thorough literature search of solid phase extraction applications to chlorophenols and phenoxy acid analysis of water was conducted, evaluated and reported. The sources searched were Chemical Abstracts, current journals and manufacturer's application notes. The information gleaned from the literature search was used to plan the subsequent stages of the study.

2. The optimum solid phase and eluting solvent combination was determined. The differences between the phases

was not as pronounced as had been anticipated, the C-18 and phenyl modified silica adsorbents were approximately equivalent with respect to the efficiency of recovery of the chlorophenols and phenoxy acids studied, and the cyclohexyl and cyanopropyl were marginally poorer.

Iso-octane was not sufficient to elute the majority of components satisfactorily. Ethyl acetate was superior and was used for the balance of the study.

3) The breakthrough volumes of the various chlorophenols and phenoxyacids were investigated on the different phases. It was found that 100 ml of surface water could be passed through the 100 mg column and still recover most analytes satisfactorily volumes of 300 ml did not appear to wash additional material off the column.

4) Solid phases manufactured by different companies were compared and the most efficient were selected. Significant variation between manufacturers exists with respect to efficiency of recovery of chlorophenols and phenoxy acids for similar adsorbents.

5) The recoveries at different concentrations and in different aqueous matrices (low and high humic acid content, river and tap waters) were determined. Recoveries were generally reproducible over a range of concentrations and a variety of surface and potable waters.

6) The volume of different water samples, that could

be passed through the column before plugging occurred, was determined. Volumes greater than 100 ml of water caused significant slowing of the flow rates, volumes above 200-300 mls appear impractical for the 100 mg tube.

With further study and improvement this should prove to be a rapid reliable method for extracting and concentrating these compounds. Attempts are currently being made to implement this method in MOE laboratories.

DIOXINS AND FURANS IN TOXIC PRECIPITATION SAMPLES

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A wide range of organic compounds have been found to exist in locations far removed from their probable sources(1). It is assumed that long range atmospheric transport and subsequent deposition via preciptiation is the mechanism responsible for the environmental spread of these compounds. This mechanism could account for the low background levels of a variety of organic compounds found in isolated locations.

Low level chlorinated dibenzo-p-dioxins (CDD's) and chlorinated dibenzofurans (CDF'S) have been found in samples taken from remote areas(2). The long range transport of CDD's and CDF's and their subsequent deposition via atmospheric precipitation has been studied at the Ontario Ministry of the Environment.

The sample collection site chosen was Dorset, Ontario (southwest of Algonquin Provincial Park). Two sample collection methods were used in this study. The first method involved the use of a custom-designed heated sampler for the direct collection of precipitation samples into pre-cleaned five gallon jugs. In the second method, an XAD-2 cartridge was used in the same sampler and the precipitation was allowed to percolate through the XAD-2 resin into a

collection jug (See Figure 1). The sampler opened only during precipitation events (rain and snow). The precipitation was collected over a four week period. The samples were then transported to the Rexdale Laboratory, solvent extracted, subjected to the Dow column cleanup method and analyzed by GC/MS for the presence of the full range of dioxin and furan congeners. To determine if any losses occurred during the handling and collection periods, the sample bottles or cartridges were spiked using ^{13}C-labelled congeners prior to collection. The samples were also spiked prior to extraction with a different set of ^{13}C-isomers to allow the determination of percentage recoveries of the CDD/CDF through the analytical processing.

Sample collection was carried out in Dorset over an eight month period. In the initial stages, direct collection of rainwater into five gallon jugs was the only sample collection method used. Later, direct collection and XAD collection methods were used in parallel. The water that percolated through the XAD was collected in a jug and also analyzed for spike and/or native CDD/CDF breakthrough from the cartridge. A spiked field blank sample was always used to determine losses that may have occurred during handling and storage. In the sets of samples analyzed, no 4CDD or 4CDF (the most toxic congeners) were found at limits of detection of 4 to 30 parts per quadrillion (ppq). Positive 8CDD was found in three monthly samples in the 60-1000 ppq range (Nov/Dec'86, March/April'87 and July/Aug'87). Lower levels of 7CDD, 7CDF and 8CDF were detected in the Nov/Dec'86 samples and a very low level of 6CDF was found in the March/April'87 sample. Field spike recoveries ranged from 10-110% and lab spikes (spiked prior to extraction) ranged from 35 to 130%. Corrections were made for lab spike recovery levels. Not enough data has been collected to see whether there are any seasonal affects.

A wall loss study was also initiated due to the 1-2 month delay between the initiation of field sampling and sample extraction. Triplicate four litre water samples were spiked with ^{13}C-4CDD and ^{13}C-8CDD at both a low and high spike level. The samples were spiked again just prior to extraction with ^{13}C-5CDD, ^{13}C-6CDD and ^{13}C-7CDD to allow calculation of extraction recoveries. The samples were extracted at the following times: 0-weeks, 6-weeks, 12-weeks and 24-weeks. Consistent recovery of the ^{13}C-4CDD at both high and low levels was obtained; however, at high spike levels of ^{13}C-8CDD there was a decrease in percentage recovery with an increase in storage time. Inconsistent recoveries were obtained for the ^{13}C-8CDD at low spike levels due to the fact that the amount spiked was close to the detection limit. This could account for the intermittent detection of the 8CDD in the precipitation samples. A future wall loss study has been initiated and results will be published elsewhere.

The presence of higher congener CDDs and CDFs in three sets of precipitation collection results from an isolated Ontario location indicates that long range transportation and deposition of airborne CDD/CDF does occur. This could account for the low background levels of 8CDD and 8CDF that are found in areas far removed from sites where dioxins are known to exist.

Future research involves the collection of precipitation samples in urban areas to determine the levels of CDD/CDF that are being deposited near industrial areas.

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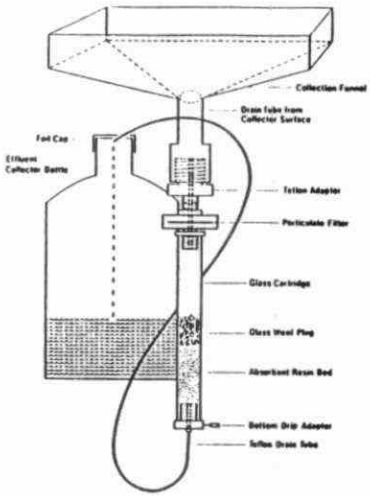
2. J.M. Czuczwa, B.D. McVeety and R.A. Hites; **Chemosphere**, 14(1985) 623-626.

Figure 1 SET UP FOR THE SAMPLING OF ORGANICS IN PRECIPITATION



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